Estrogen Receptor-nuclear Matrix Interactions In The Female Mouse Brain.

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Estrogen Receptor-Nuclear Matrix Interactions in the Female Mouse Brain

by

Suzanne E. McKenna

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May 9, 1996
Accepted Date

Committee Members:

Neal G. Simon, Ph.D.
committee chair

Michael Kuchka, Ph.D.
Committee Member

Evelyn Barrack, Ph.D.
Committee Member

Anne Etgen, Ph.D.
Committee Member

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Abstract

The nuclear matrix (NM), the insoluble skeletal framework of the nucleus, has been implicated in the regulation of gene expression due to its role in DNA organization, replication, and transcription, the preferential association of actively transcribed genes, transcription factors and steroid receptors (SR). Steroid receptors associate with the NM following *in vivo* and *in vitro* exposure to hormones and these interactions are saturable, of high affinity, and tissue- and steroid-specific. The colocalization of transcriptionally active genes and SR on the NM makes the NM a strong candidate for the functionally significant nuclear binding site for the steroidal regulation of hormone-responsive genes. Remarkably, gonadal SR-NM interactions have never been assessed in neural tissue. To address this issue a procedure for measuring estrogen receptor (ER)-NM interactions in the central nervous system of female mice was developed.

NM was prepared from limbic tissue (a region rich in ER) of female mice (60-70 days of age) treated with estradiol benzoate (EB) or oil vehicle. ER-NM interactions were assessed biochemically by exchange assay and immunochemically by Western blot. Exchange

assays revealed the presence of saturable, high affinity (K_d = 1.15 x 10^{-9} M) [^{3}H] E binding sites in limbic NM that were dependent upon the presence of EB *in vivo*. This was confirmed by Western blot analysis which revealed three immunoreactive bands of 46, 68, and 97 kDa present in NM from EB-treated females that were essentially absent in the NM of oil-treated mice. The 68 kDa band indicated the presence of ER, the 46 kDa species presumably represented a proteolytic degradation product, and the 97 kDa band may indicate ER associated with a component of the NM, or a previously unidentified ER species. The biochemical and immunochemical data are consistent with previous reports of the hormone-dependent association of SR with NM of peripheral target tissues.

The documentation of ER-NM interactions in neural tissue establishes a level of analysis for the physiological actions of steroid hormones previously unavailable in neural tissue which could potentially provide insights into the molecular mechanisms involved in the establishment of sexual dimorphisms in steroid hormone sensitivity in the brain.

CHAPTER ONE: INTRODUCTION

Steroid Hormone Receptors

Steroid hormones play a central role in developmental and physiological regulation in a wide range of species. The gonadal steroids (estrogens, progestins, and androgens) regulate the development and determination of the embryonic reproductive system, the differentiation of accessory reproductive organs and external genitalia, masculinization/feminization of the brain, and the development and expression of reproductive behaviors (Harris, 1964; Jost, 1972; Pfeiffer, 1936; Phoenix, Goy, Gerall, and Young, 1959; vom Saal, Montano, and Wang, 1992; Wilson, George, and Griffin, 1981). Hormonal signals are mediated via hormone-specific receptors which are localized predominantly in the nucleus of target tissues, i.e., tissues that respond to hormonal stimulation. The first steroid receptor identified was the estrogen receptor (ER) (Glascock and Hoekstra, 1959; Jensen and Jacobson, 1960; 1962; Jensen, Sujuki, Kawashima, Stumpf, Jungblut, and DeSombre, 1968; Toft and Gorski, 1966). This discovery was soon followed by the isolation of a number of other receptors including those for progestins, androgens, thyroid hormones, glucocorticoids, mineralocorticoids, retinoids, and

vitamin D. Together these receptors comprise a supergene family known as the nuclear receptor superfamily, the members of which may have evolved from a single common ancestral gene (Amero, Kretsinger, Moncrief, Yamamoto, and Pearson, 1992; Beato, 1989; Evans, 1988; Green and Chambon, 1988; Tsai and O' Malley, 1994). All members of the superfamily contain structurally and functionally distinct domains including i) the N-terminal region of the receptor which houses one of the regions responsible for transcriptional activation (activation function 1; AF1); ii) the deoxyribonucleic acid (DNA)-binding domain; and iii) the hormone-binding domain in which AF2 is localized (Bocquel, Kumar, Stricker, Chambon, and Gronemeyer, 1989; Jensen, 1991). In addition to the aforementioned receptors, there are also more than 40 "orphan receptors" of unknown function which are structurally similar to the members of the nuclear receptor superfamily, for which ligands have not yet been identified (Giguere, Yang, Segui, and Evans; 1988; O'Malley 1989; Wang, Tsai, Cook, Beattie, Tsai, and O'Malley, 1989).

In the three decades that have followed since the identification of the ER, many of the intricacies of the mechanism of action of steroid hormones and their receptors have been elucidated. Past studies have examined aspects of receptor function including

structure, ligand binding, as well as interactions with various cellular components including transcription factors and DNA (Beato, 1989; Evans, 1988; Gorski, Furlow, Murdoch, Fritsch, Kaneko, Ying, and Malayer, 1993; Green and Chambon, 1988; Katzenellenbogen, 1980; Landers and Spelsberg, 1992; Malayer and Gorski, 1993; O'Malley and Tsai, 1992; 1993; O'Malley, Tsai, Bagchi, Weigel, Schrader, and Tsai, 1991; Tsai and O'Malley, 1994; Truss and Beato, 1993; Yamamoto, 1985). The current model of genomic steroid action is depicted in Figure 1. Steroid hormones are lipid soluble molecules that freely diffuse across the cell membrane. Target cells possess receptors for these hormones which reside primarily in the nuclear compartment for most classes of steroid hormones (Hansen, Welshons, and Gorski, 1988; King and Greene, 1984; Willmann and Beato, 1986). These receptors are steroid- and tissue-specific and are present at the level of 15,000-20,000 per cell (Clark and Peck, 1976; Katzenellenbogen, 1980). Steroids associate with their receptors in a reversible fashion and bind with high affinity, in the range of 10^{-8} - 10^{-10} M (Clark and Peck, 1977). Steroid binding results in receptor activation/transformation due to a conformational shift in the structure of the receptor concomitant with the dissociation of non-steroid binding receptor-related proteins, e.g.,

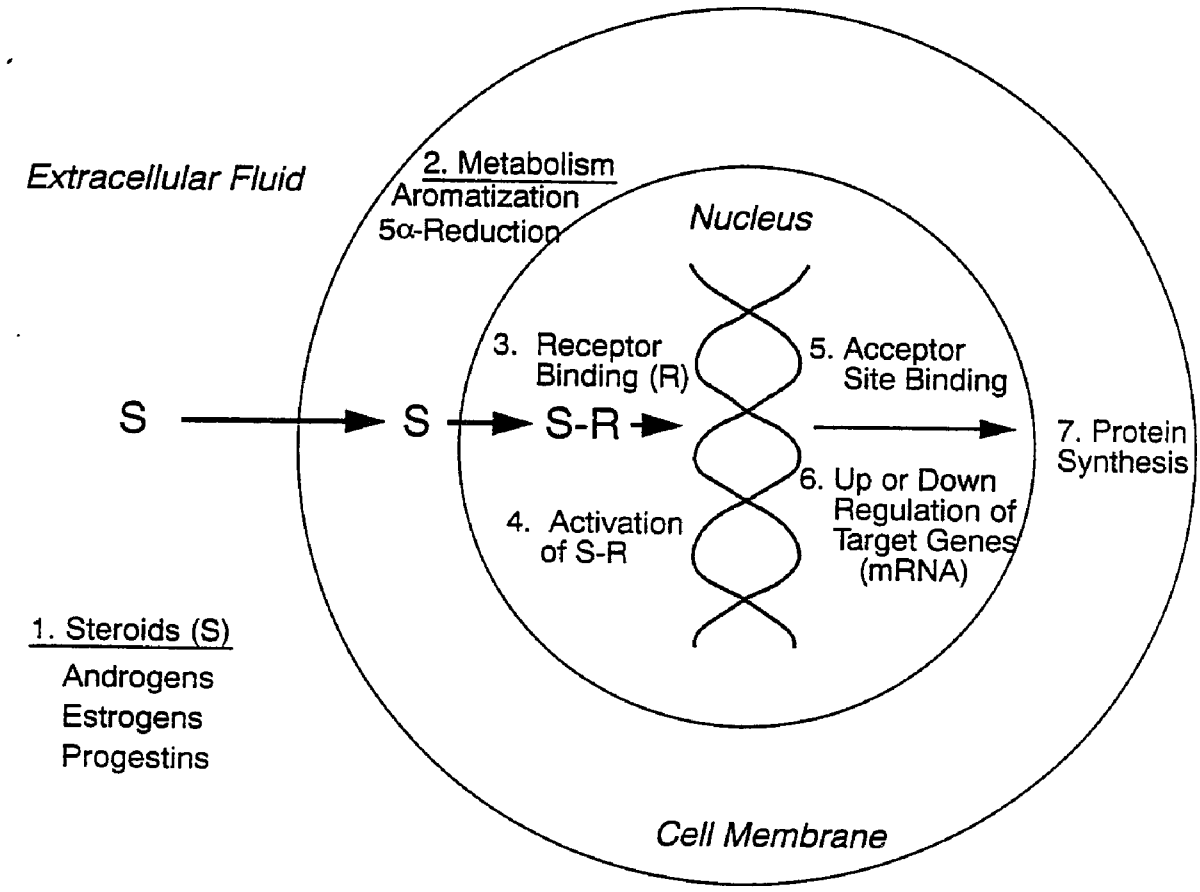


Figure 1. Genomic mechanism of action of steroid hormones.

heat shock proteins (Beato, 1989; Fritsch, Leary, Furlow, Ahrens, Schuh, Mueller, and Gorski, 1992; Hansen et al., 1988; Hansen and Gorski, 1985; Malayer and Gorski, 1993; Pratt, 1990; 1993; Pratt, Hutchinson, and Scherrer, 1992). Receptor activation is required for high affinity binding of the steroid receptor to its steroid response element (SRE), a sequence of DNA specific for a given receptor, resulting in an alteration in the level of gene transcription and/or modification of posttranscriptional steps altering the levels of messenger ribonucleic acid (mRNA) and subsequently protein synthesis (Beato, 1989; Fritsch, et al., 1992; Hansen et al., 1988; Hansen and Gorski, 1985; Landers and Spelsberg, 1992; Lucas and Granner, 1992; Malayer and Gorski, 1993; Truss and Beato, 1993; Yamomoto, 1985). In this manner, steroid receptors function as ligand-inducible transcription factors that interact with specific DNA sequences (SREs) in the promoter region of target genes resulting in quantitative shifts in mRNA production and protein levels.

Elegant *in vitro* experiments utilizing overexpressed and/or recombinant receptor preparations and linear segments of DNA containing SREs have resulted in extensive characterization of SR-SRE interactions (Beato, Barretino, Bruggemeier, Chalepakis, Hache, Slater, and Truss, 1991; Gronemeyer, 1993; Martinez, Givel, and Wahli,

1987; Martinez and Wahli, 1991; Truss and Beato, 1993; van der Ahe, Janich, Scheidereit, Renkawitz, Schutz, and Beato, 1985). However, these experiments do not take into account the complex structural environment of the genome *in vivo* , and the levels of regulation such a structure affords (for review see Felsenfeld, 1992; Grunstein, 1990; Hager and Archer, 1991; Latchman, 1990; Mirkovitch, Gasser, and Laemmli, 1987; Owen-Hughes and Workman, 1994). A discussion of DNA organization and its contribution to transcriptional regulation is discussed below.

DNA Organization: Effects on Transcriptional Regulation

In eukaryotic cells, approximately 2 meters of DNA is condensed by 200,000-fold so that it may fit within the confines of the nucleus. Condensation is achieved by compaction of DNA into several hierarchical structures: the nucleosome, the 30 nm chromatin filament, and loop domains as depicted in Figure 2 (reviewed in Getzenberg, Pienta, Ward, and Coffey, 1991; Pienta, Getzenberg, and Coffey, 1991; van Holde, 1989; Wolffe, 1992). Nucleosomes are composed of an octamer of histone proteins around which is wound

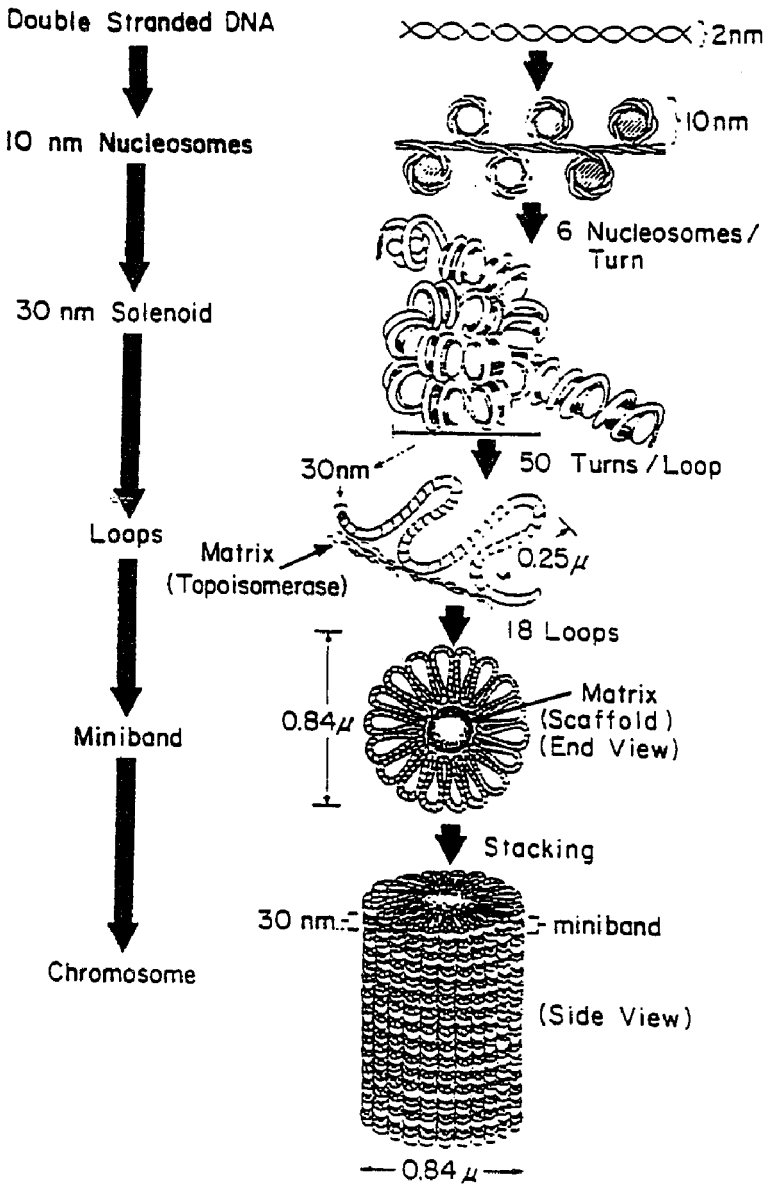


Figure 2. Schematic of the levels of organization of DNA. Reprinted from Getzenberg, Pienta, Ward and Coffey, 1991.

160 base pairs of DNA. Arrays of nucleosomes are wound into a solenoid structure with 6 nucleosome particles per turn to form the 30 nm chromatin filament. Condensation of the 30 nm chromatin filament is achieved via the formation of roughly 50,000 loop domains each consisting of some 60 kilobase pair loops of DNA that are attached at their bases to the nuclear matrix (NM- the insoluble skeletal framework of the nucleus). In the chromosome these loops are dispersed radially, 18 loops per turn, forming the miniband. The minibands are wound and stacked along a central axis to form each chromatid of the chromosome (Felsenfeld and McGhee, 1986; Getzenberg, et al., 1991; Mirkovitch, Mirault, and Laemmli, 1984; van Holde, 1989; Wolffe, 1992: Zehnbauer and Vogelstein, 1985).

Progressive compaction of DNA limits the accessibility of *trans*-acting factors (e.g., transcription factors) to *cis*-acting sequences (e.g., DNA binding sites) involved in transcription. For example, chromatin-mediated gene suppression has been identified in a number of systems including expression of the PHO5 gene in *Saccharomyces cerevisiae* and the mouse mammary tumor virus in response to the presence of phosphate and glucocorticoids, respectively (Hager, Richard-Foy, Kessel, Wheeler, Lichtler, and Ostrowski, 1984; Straka and Horz, 1991). In these two systems,

uniquely positioned nucleosomes repress transcription until the appropriate *trans*-acting factor binds to its cognate *cis*-acting DNA element and displaces the nucleosome. Nucleosome displacement results in increased accessibility of a previously sequestered *cis*-acting sequence, thereby relieving transcriptional repression. In light of data such as these, analyses of steroid regulated gene expression must take into account the structural environment of the genome *in vivo* and the extent to which this may modulate the activation/repression of transcription. To this end, a great deal of research has focused on the NM, which is described in detail below.

The Nuclear Matrix

The NM, first described by Berezney and Coffey (1974), is the insoluble skeletal framework of the nucleus that is resistant to extraction with detergent, DNase, and salt. It consists of the peripheral lamina and pore complexes, an internal ribonucleic protein network, and residual nucleoli (Berezney and Coffey, 1974; 1975; 1976). The NM retains approximately 10-20 % of the total nuclear protein, 2-10 % of the total nuclear DNA and 30% of the RNA (Barrack, 1987a; Barrack and Coffey, 1982; Berezney and Coffey,

1974, 1975, 1976; Nelson, Pienta, Barrack, and Coffey, 1986). This structure is intimately involved in a number of biologically significant functions including DNA organization, replication, and transcription, and more recently, macromolecular transport within nuclei (Barrack and Coffey, 1982; Berezney, 1991; Berezney, Basler, Bucholtz, Smith and Siegel, 1982; Berezney and Coffey, 1974, 1975, 1976; Bonifer, Hecht, Saueressig, Winter, and Sippel, 1991; Fey, Bangs, Sparks, and Odgren, 1991; Getzenberg, Pienta, and Coffey, 1990; Getzenberg, 1994; Nelson, et al., 1986; Nickerson, He, Fey, and Penman, 1990; Razin and Gromova, 1995; Shaper, Pardoll, Kaufman, Barrack, Vogelstein, and Coffey, 1979; Stein, van Wijnen, Stein, Lian, Bidwell, and Montecino, 1994; Sun, Chen and Davie, 1994; Vogelstein, Nelkin, Pardoll, and Hunt, 1982; Wanka, Pieck, Bekers, and Mullenders, 1982). Newly synthesized RNA transcripts have been localized to the NM and active genes are associated with the NM only in cell types in which they are expressed; examples of NM-associated genes are provided in Table 1 (Andreeva, Markova, Loidl, and Djondurov, 1992; Berezney, 1991; Buttyan and Olsson, 1986; Carter, Bowman, Carrington, Fogarty, McNeil, Fay, and Lawrence, 1993; Ciejek, Tsai, and O'Malley, 1983; Ciejek, Nordstrom, Tsai, and

Tissue/Cell Type	Gene
rat liver	ribosomal RNA
rat liver	α_2-macroglobin
rat ventral prostate	prostatein C-3
rat seminal vesicle	SVS-IV
NIH 3T3 cells	SV40
HeLa cells	newly transcribed sequences
human HL-60	c-*myc*
human skin	proα2(I) collagen
human erythrocytes	globin
human lymphocytes	immunoglobulin
mouse lymphocytes	α-globin
chicken oviduct	ovalbumin
chicken liver	vitellogenin II
chicken erythrocytes	β-globin
chicken	lysozyme
Drosophila	heat shock
nine cell types	polyoma and avian sarcoma viruses

Table 1. Active genes associated with the nuclear matrix from tissues expressing these genes and absent from non-expressing tissues.

O'Malley, 1982; Fey et al., 1991; Getzenberg, et al., 1991; Huang and Spector, 1991; Jackson, McCready, and Cook, 1981; Jost and Seldran, 1984; Nelson et al., 1986; Robinson, Nelkin, and Vogelstein, 1982; Smith, Harris, Zillmann, and Berget, 1989; Thorburn, Moore, and Knowland, 1988; Xing, Johnson, Dobner, and Lawrence, 1993). In addition, steroid receptors and transcription factors (e.g., SP-1, OCT-1, NF-1, and AP-1) preferentially associate with the NM (Bidwell, van Wijnen, Fey, Dworetzky, Penman, Stein, Lian, and Stein, 1993; Boulikas, 1993; 1994; Dworetzky, Fey, Penman, Lian, Stein, and Stein, 1992; Stein, Lian, Dworetzky, Owen, Bortell, Bidwell, and van Wiijnen, 1991; Sun, Chenm abd Davie 1994; van Wijnen, Bidwell, Fey, Penman, Lian, Stein, and Stein, 1993; Vassetzky, De Maura Gallo, Bogdanova, Razin, and Scherrer, 1993).

Protein Constituents of the Nuclear Matrix

Due to the many biological functions associated with the NM, a great deal of research has been directed towards understanding its structural components. Recent research has focused on two dimensional electrophoretic analysis of NM proteins (NMP) resulting in two classifications. The first class represents the majority of NMP

that are found in a large proportion of mammalian cells (Fey et al., 1991; Getzenberg, 1994: Stuurman, Meijne, van der Pol, de Jong, van Driel, and van Renswolde, 1990; Nakayasu and Berezney, 1991; Partin, Getzenberg, CarMichael, Vindivich, Yoo, Epstein, and Coffey, 1993). These likely represent the fraction of proteins involved in the basal functioning of the NM and include the majority of high molecular weight nonhistone proteins including actin, lamins A, B, and C, nuclear matrins E, F, G and 4 (Berezney, 1991; Fey et al., 1991; Nakayasu and Berezney, 1991). The second class of NMP are cell type-, tissue-, malignancy-, and differentiation state-dependent (Berezney, 1991; Brasch and Peters, 1985; Dworetzky, et al., 1992; Fey and Penman, 1988; Getzenberg and Coffey, 1990; Getzenberg et al., 1991; Kallajoki and Osborn, 1994; Khanuja, Lehr, Soule, Gehani, Noto, Choudhury, Chen, and Pienta 1993; Mattia, Eufemi, Chichiarelli, and Ferraro, 1995; Partin et al., 1993; Pienta and Lehr, 1993; Sauermann, Korosec, and Gerner, 1995; Stuurman, van Driel, de Jong, Meijne, and van Renswolde, 1989; Stuurman, Meijne, van der Pol, de Jong, van Driel, and van Renswolde, 1990). It is the existence of this second class that provides compelling evidence for participation of the NM in the regulation of gene expression.

Several investigators have suggested that tissue-specific NMP composition may be responsible for establishing a unique 3-dimensional organization of DNA, resulting in, for example, the expression of a specific gene in one tissue, but not in another (reviewed in Getzenberg, 1994). An indication of the role of NMPs in the regulation of gene expression comes from the finding that the acquisition of a transformed phenotype is correlated with specific changes in NMP content. Changes in NMP content were correlated with the progression from normal to intermediate to the transformed phenotype in both breast and prostate cancer samples (Khanuja et al., 1993; Partin et al., 1993; Pienta and Tracy, 1995). In the prostate, modifications in NMPs were discovered that were specific to patients with benign prostatic hyperplasia (BPH) and a second set of differences in NMPs were detected in patients with prostate cancer (Partin et al., 1993; Pienta and Replogle, 1995). Thus BPH may represent an intermediate state during the progression from the normal to the cancerous phenotype, and changes in NMP composition paralleled this progression. Similar findings were reported following an examination of NMPs from normal breast tissue samples, MCF-10A cells (a spontaneously immortal human breast epithelial cell line that expressed both normal and cancer tissue-specific NMPs but was

not tumorigenic in nude mice) and cancerous breast tissue samples (Khanuja et al., 1993). Recently Pienta and Replogle (1995) reported the existence of two groups of NMPs in breast cancer cells; one set was found only in normal breast epithelial cells and the other set was expressed only in breast cancer cells. Therefore, alteration in NMP expression, which may modulate DNA organization, could presumably play a role in the alteration of gene expression characteristic of the transformed state (Pienta and Lehr, 1993; Pienta and Replogle, 1995; Getzenberg, et al., 1991).

Further evidence for participation of the NM in the regulation of gene expression is derived from analyses of NMPs during the development and maintenance of the osteoblast phenotype during the establishment of bone tissue organization *in vitro* (Stein, Stein, Lian, Montecino, and van Wijnen, 1995; Stein, et al., 1994). Changes in the protein composition of the NM throughout the osteoblast developmental sequence paralleled changes in gene expression. When the developmental sequence was delayed *in vitro*, the NM retained the stage-specific NMP composition associated with the stage in which the developmental delay occurred (Dworetzky, et al., 1990; Stein, et al., 1994). It seems that there is a clear correlation between the protein constituents of the NM and cell-, tissue-,

malignancy- and differentiation state-dependent gene regulation. More research will be necessary to further characterize this link.

Another variable that affects NMP content is hormonal status (Coutts, Davie, and Murphy, 1995; Getzenberg, 1994; Getzenberg and Coffey, 1990). Getzenberg and Coffey (1990) investigated NMP content in two androgen-dependent tissues in the rat, prostate and seminal vesicle. Each tissue synthesizes secretory protein(s) not produced by the other, and this tissue-specific protein expression was correlated with the localization of the corresponding gene on the NM only in the tissue in which it was expressed (i..e., C3 on prostate NM and SVS IV on seminal vesicle NM). In addition, each tissue had a specific NMP composition as examined via 2D- electrophoresis. Following castration, neither tissue synthesized its tissue-specific secretory protein and both qualitative and quantitative changes in NMP components were noted. Three proteins appeared and none disappeared in prostate NM, whereas four proteins appeared and two proteins disappeared in seminal vesicle NM following castration (Getzenberg and Coffey, 1990). Thus hormone-dependent changes in NMP composition were correlated with changes in gene expression following castration. Steroid mediated changes in NMP content have been reported *in vitro* as well. For example, estrogen-regulated

NMPs in T47D-5 human breast cancer cells have recently been reported (Coutts et al., 1995). The levels of three proteins present in the NM of cells grown in the presence of estrogen were dramatically reduced following one week of estrogen withdrawal. Replacement of estrogen in the culture medium restored the levels of these proteins, while the addition of tamoxifen resulted in a significant reduction (Coutts et al., 1995). The role of steroid hormones in mediating DNA organization by modulating NMP content will be important to resolve.

The presence of cell-, tissue-, malignancy-, differentiation state-, and hormone-dependent NMP components and their correlation with changes in gene expression point to the participation of the NM in gene regulation. Figure 3 depicts a model proposed by Pienta and colleagues (1991) to explain the role of tissue-specific DNA organization in gene expression. In this model, tissue-specific NMPs are involved in the binding and localization of specific DNA sequences (i.e., an active gene) on the NM through the formation of DNA loop domains. This interaction results in the positioning of genes in the proper three-dimensional configuration for transcription factor interaction, and in some cases colocalizes *cis*-acting DNA sequences with cognate transcription factors that are bound to the

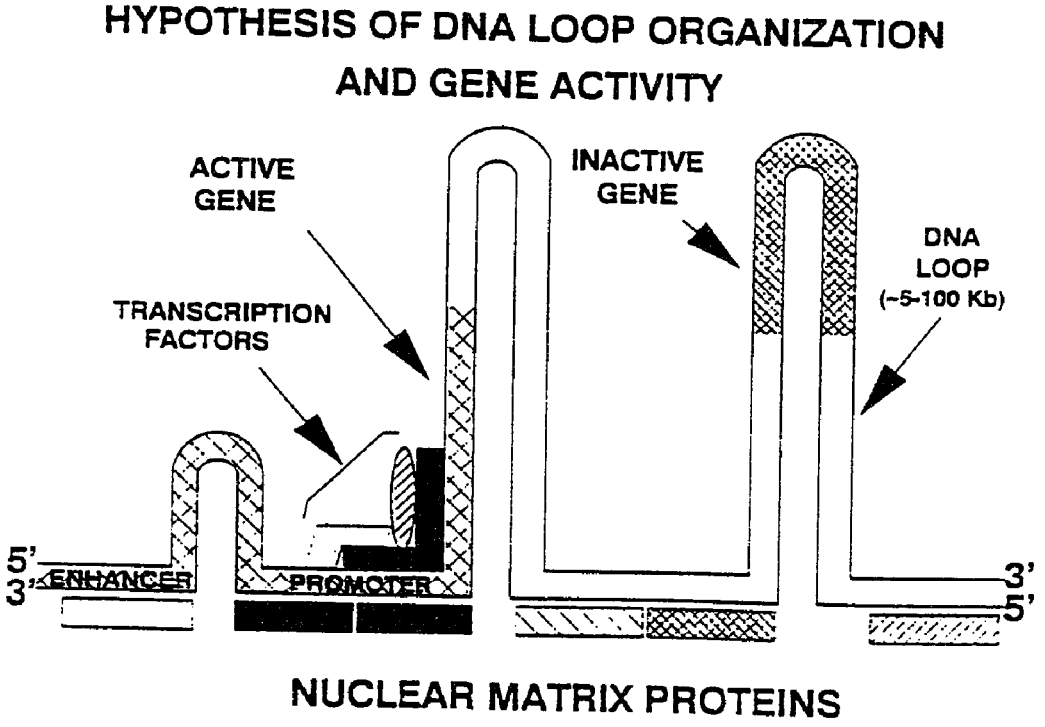


Figure 3. The role of tissue-specific DNA organization in gene expression. Active genes are associated with the nuclear matrix. Tissue-specific nuclear matrix proteins (NMPs) are involved in the binding and localization of specific DNA sequences and determining the proper 3-dimensional context for transcription factor interaction. Reprinted from Getzenberg, Pienta, Ward and Coffey, 1991.

NM (e.g., steroid receptors). In this model, inactive genes are located peripherally in the loop domains. By providing the correct three-dimensional organization of DNA, NMPs may play a role in the regulation of gene expression. Given such a model, the existence of hormonally modulated NMPs establishes a new level at which steroid hormones may regulate gene expression and points to the importance of analyzing SR-genome interactions *in vivo* . A major focus of NM research in the past has been the association of steroid receptors (SR) with the NM. The present state of knowledge concerning SR-NM interactions, which is described below, provides compelling evidence that the NM is the physiologically significant nuclear binding site, or acceptor site, for steroid hormone-mediated gene regulation.

Steroid Hormone Receptor-Nuclear Matrix Interactions

Numerous studies have reported that SR-NM interactions are saturable, of high affinity, and tissue- and steroid-specific (Alexander, Greene and Barrack, 1987a; Barrack, 1983, 1987; Barrack and Coffey, 1980, 1982; Belisle, Bellabarba, and Lehoux, 1989; Buttyan, Olsson, Sheard, and Kallos, 1983; Colvard and Wilson, 1984;

Kaufmann, Okret, Wikström, Gustafsson, and Shaper, 1986; Kumara-Siri, Shapiro, and Surks, 1986; Metzger, Curtis, and Korach, 1991; Metzger and Korach, 1990; Mowszowicz, Doukani, and Giacomini, 1988; Rennie, Bruchovsky, and Cheng, 1983; Swaneck and Alvarez, 1985; van Steensel, van Haarst, de Kloet, and van Driel, 1991). A list of SR associated with NM of various tissues is given in Table 2. The fraction of nuclear receptors bound to the NM represents the majority of total nuclear receptors, in the range of 40-98% for various tissues (Barrack, 1987a). This wide range of values, as depicted in Table 3, may be due to differences in endogenous protease activity in the various tissues examined, in addition to methodological differences. For example, the extent of disulfide bond formation either inadvertently or deliberately introduced *in vitro* significantly affected the proportion of nuclear glucocorticoid receptors that remained associated with the NM (< 5 to > 95%; Kaufman et al., 1986). Methodological differences notwithstanding, on average, 63% of the nuclear receptors were localized in the NM and these receptors were thought to be the same population described as the "salt-resistant" nuclear fraction -- the fraction of receptors that resist extraction from the nucleus in the presence of

Species/Tissue	Receptor Type
rat uterus	estrogen
rat ventral prostate	androgen
mouse uterus	estrogen
rat dorsolateral prostate	androgen
rat liver	thyroid
rat liver	estrogen
rat liver	glucocorticoid
rat prostate cancer (R3327G)	androgen
avian oviduct	progesterone
hen liver	progesterone
hen liver	estrogen
guinea pig seminal vesicle	androgen
human foreskin	androgen
human prostate cancer	androgen
human prostate (BPH)	androgen
rat hippocampus	corticosteroid
rat pituitary tumor cells (GC)	thyroid
COS-1 cells	androgen
COS-1 cells	glucocorticoid

Table 2. Steroid receptors that specifically associate with the nuclear matrix.

Species/Tissue	Hormone	% Nuclear
rat uterus	estrogen	62
rat uterus	estrogen	46
rat liver	estrogen	60
rat liver	glucocorticoid	60
rat liver	glucocorticoid	95
rat ventral prostate	androgen	67
rat ventral prostate	androgen	50
rat ventral prostate	androgen	78
rat prostate	estrogen	65
rat dorsolateral prostate	androgen	98
rat prostate cancer (R3327G)	androgen	69
human prostate (BPH)	androgen	50
rat pituitary tumor (GC)	T3	40
rat hippocampus	corticosterone	60
rat hippocampus	corticosterone	100
hen liver	estrogen	62

Table 3. Percentage of nuclear receptors localized on the nuclear matrix of hormone responsive tissues.

high concentrations of salt (0.6 M NaCl or KCl) (Barrack, 1987a; Barrack and Coffey, 1982; Barrack, Hawkins, Allen, Hicks, and Coffey, 1977; Baudendistel and Ruh, 1976; Clark and Peck, 1976; Mester and Baulieu, 1975; Metzger and Korach, 1990; Ruh and Baudendistel, 1977; Sato, Spomer, Huseby, and Samuels, 1979; Simmen, Means and Clark, 1984). The number of salt-resistant receptors has been correlated with the number of receptors necessary for biologically significant events and are thought to represent receptors associated with the nuclear acceptor sites (Baudendistel and Ruh, 1976; Clark and Peck, 1976; Clark, Williams, Upchurch, Eriksson, Helton, and Markaverich, 1982; Markaverich, Upchurch, and Clark, 1981; Mester and Baulieu, 1975; Ruh and Baudendistel, 1977).

One example of the correlation between biological responsiveness and the presence of salt-resistant receptors was found in a study of two strains of mice that exhibited a tissue-specific sensitivity to estrogen. Balb/c mice, which were sensitive to estrogen-induced testicular neoplastic transformation, exhibited a greater degree of salt-resistant nuclear ER than C_3H_{Bi} (Z) mice, which were tumor-resistant (Sato, et al., 1979). Although Balb/c mice possessed significantly greater quantities of ER compared to Z mice, cell-free cross-over experiments indicated that the result of the salt-

extraction experiment was due to differences in chromatin rather than E-ER complexes (Sato, et al., 1979). The number of salt-resistant nuclear ER also correlated with the ability of estrogen to induce DNA polymerase α in the testes of these two strains of mice; estrogen induced polymerase α in the testes of BALB/C, but not Z mice (Spruance, Wilcox, Richards, Foster, Huseby, and Samuels, 1978). However, uteri from females of both strains exhibited essentially the same degree of salt-resistant ER (Sato et al., 1979).

In addition, the biological activity of stilbestrol estrogens correlated with the number of nuclear salt-resistant ER (Metzger, et al., 1991). Indenestrol-1A and z-pseudo-diethylstilbestrol both exhibited similar levels of nuclear binding but different levels of salt-resistant binding, 61% and 29%, respectively. The amount of salt-resistant binding paralleled the ability of these compounds to elicit some estrogenic responses. Therefore, the functional status of these estrogenic compounds was correlated with the resistance of the ligand-bound ER to extraction with salt.

Another example can be found in studies that correlated the number of salt-resistant ER with the number of ER necessary for uterine growth. Anderson, Clark and Peck (1972;1975) reported that the long term retention (6 hours) of approximately 1,000-3,000

estrogen receptor (ER) per uterine cell must be maintained for maximal uterine growth to occur. Injections of 2.5 and 0.1 μg estradiol both induced maximal uterine growth and resulted in 20 and 60% of nuclear ER exhibiting salt-resistance, respectively (Clark and Peck, 1976). Although the percentage of salt-resistant ER was dependent on the dose of estradiol administered, the actual number of salt-resistant ER was the same in both cases (1400 ER/uterine cell; Clark and Peck, 1976). These findings on the number of salt-resistant nuclear ER present following injection of 0.1 μg estradiol were corroborated by Barrack and colleagues (1977).

Based on these observations, it seems that only a fraction of SR is necessary for the regulation of a target gene, as has been suggested by others (Clark and Peck, 1976; Katzenellenbogen, 1980; Simmen, et al., 1984). As described above only 1,000-3,000 ER per cell were required for uterine growth despite the presence of 15,000-20,000 ER per uterine cell (Clark and Peck, 1976). A more recent example has been provided by Lubahn and colleagues resulting from the creation of mutant mice containing an insertional disruption in the ER gene (Lubahn, Moyer, Golding, Couse, Korach, and Smithies, 1993). Mice homozygous for this mutation lacked functional uterine ER, whereas heterozygous mice had approximately

50% of uterine ER present in wild type (WT) mice (Couse, Curtis, Washburn, Lindzey, Golding, Lubahn, Smithies, and Korach, 1995). Despite this large deficit in uterine ER, heterozygous mice exhibited increases in uterine wet weight and hyperemia in response to E at a level equivalent to WT, providing evidence that the full complement of receptors was not required for these functional responses to estrogen (Couse et al., 1995).

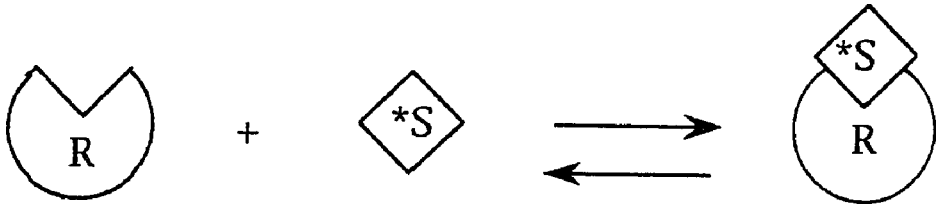
In regard to the central nervous system, support for this concept can be found in a study that measured ER and progesterone receptor (PR) concentrations in various brain regions of E-treated female rats (Brown, MacLusky, Shanabrough, and Naftolin, 1990). The authors reported an age-dependent decrease in the concentration of ER in the pituitary and hypothalamus that was not accompanied by a decrease in the concentration of PR, a protein whose induction is regulated by ER. If the full complement of ER present in young rats was necessary to induce the observed concentration of PR, one would expect to see a decline in PR induction as ER concentration declined with age. However, this did not occur. Therefore, it seems that not all of the ER present were necessary for maximal PR induction. Findings such as these call into question the practice of simply quantifying changes in the overall concentration of

receptors and indicate that an important step will be to measure the concentration of receptors that are biologically significant, presumably those associated with the nuclear acceptor site. A great deal of evidence, which is presented below, supports the role of the NM as the nuclear acceptor site.

The Nuclear Matrix as Acceptor Site

Analyses of SR-NM interactions have utilized both receptor and acceptor assays. As depicted in Figure 4, receptor assays involve the *in vitro* incubation of a constant amount of receptor preparation with increasing concentrations of radiolabeled hormone, whereas acceptor assays involve the *in vitro* incubation of a constant amount of acceptor preparation with increasing concentrations of radiolabeled hormone-receptor complex (Barrack, 1987a; Metzger and Korach, 1990). Receptor/acceptor assays yield different types of information depending upon the incubation temperature utilized. Assays conducted at 0° C result in the quantitation of the number of receptors/acceptors **unoccupied** in the preparation, whereas incubation at 37° C, referred to as an exchange assay, results in

(A) RECEPTOR ASSAY



(B) ACCEPTOR ASSAY

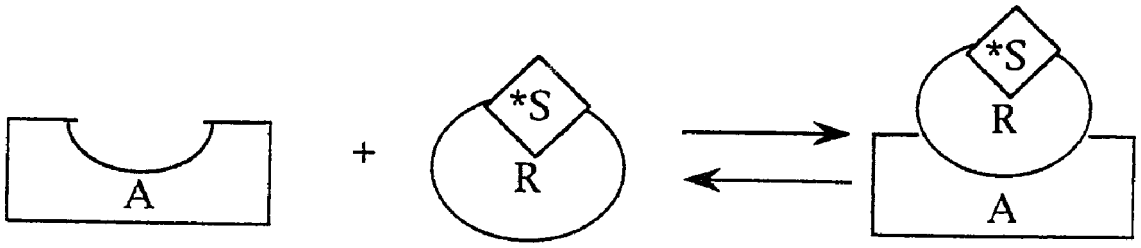


Figure 4. Schematic representation of receptor/acceptor assays. *S, radiolabeled steroid; R, receptor; A, acceptor. In receptor assays (A), receptors are quantitated by incubation of a constant amount of receptor (R) with increasing concentrations of radiolabeled steroid (S*). In acceptor assays (B), acceptors (A) are quantitated by incubation of a constant amount of acceptor with increasing concentrations of radiolabeled steroid-receptor complexes (*SR).

quantitation of the **total** number of receptors/acceptors present (Barrack, 1987a; Clark and Peck, 1977; Roy and McEwen, 1977). This distinction is depicted in Figure 5 in which the *in vitro* binding of tritium-labeled E-ER complexes ([^{3}H]E-ER) to NM prepared from estradiol-treated mice was assayed at 0° C and 37° C. Estradiol injected *in vivo* interacts with endogenous ER to form E-ER complexes that occupy the acceptor sites. NM is prepared and incubated *in vitro* with [^{3}H]E-ER under conditions that do not (0 ° C) or do (37° C) permit exchange of the endogenous E-ER for [^{3}H]E-ER. Incubation at 0° C does not permit the dissociation of the endogenous E-ER from the acceptor site, therefore the only acceptor sites available for binding by [^{3}H]E-ER are those that were not occupied *in vivo*. In comparison, incubation at 37° C does result in dissociation of the endogenous E-ER from the acceptor site, enabling them to be replaced by the [^{3}H]E-ER complexes. By assaying NM at these two different temperatures it is possible to differentiate between the number of acceptor sites available and the total number of acceptor sites in a preparation. Furthermore, this same experiment can be run with the modification that the NM is incubated with [^{3}H]E to quantitate the number of ER associated with the NM. The studies

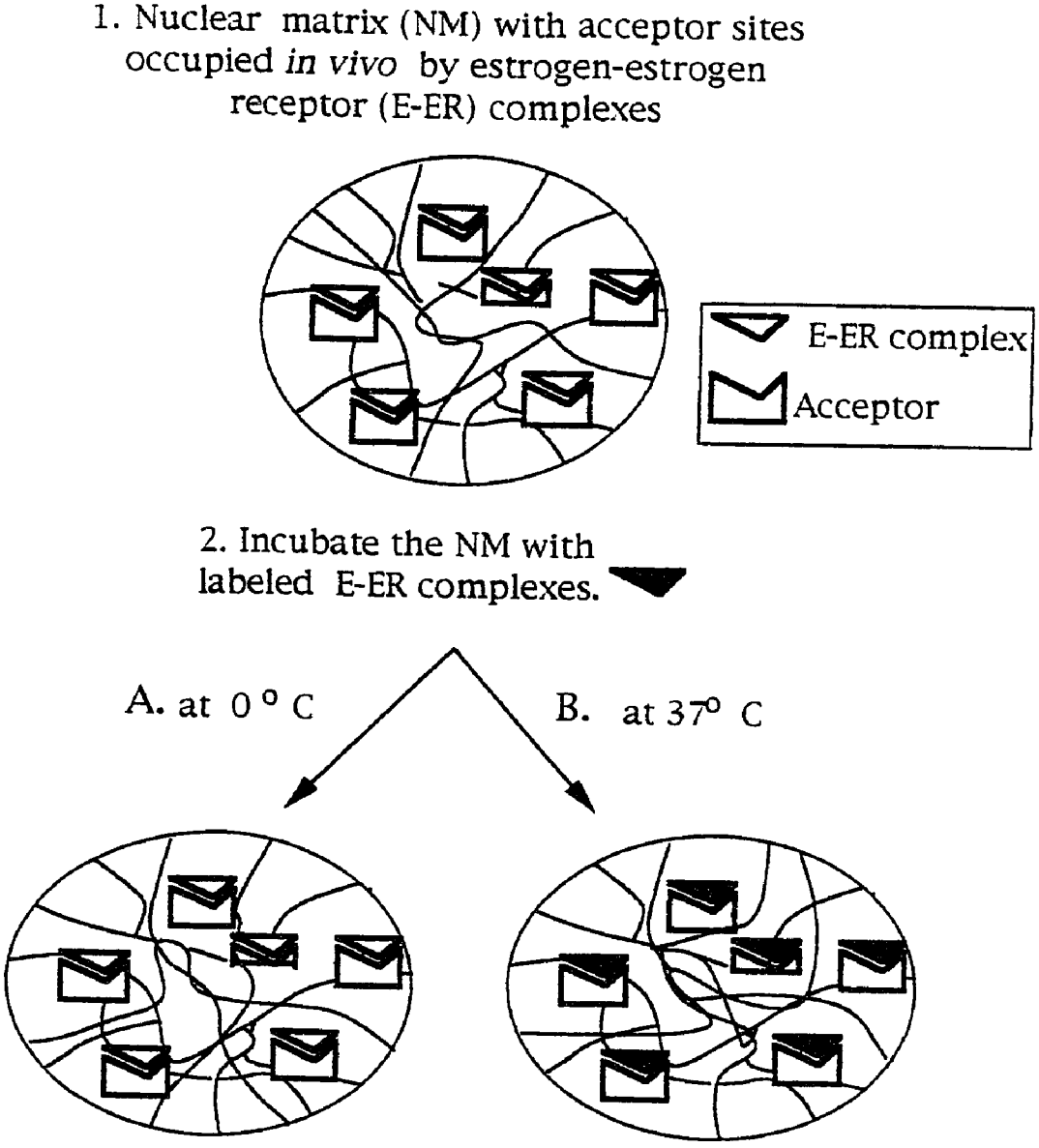


Figure 5. Acceptor assays: measurement of unoccupied versus total binding sites. A. At 0° C, endogenous E-ER complexes cannot exchange *in vitro* for the labeled E-ER complexes. Therefore, any labeled complexes that bind to the nuclear matrix are binding to acceptor sites that were unoccupied *in vivo*. B. At 37 ° C, endogenous E-ER complexes are exchanged *in vitro* for the labeled E-ER complexes. This results in the quantitation of the total number of acceptor sites present *in vivo*.

described below utilized these methods, as well as others, to establish that the NM may represent the nuclear acceptor site for SR.

Validation of the NM as the nuclear acceptor site comes from a study utilizing a cell-free assay procedure conducted under conditions that did not permit exchange comparing competitive binding by unlabeled ER for [^{3}H]ER in chromatin, DNase-treated chromatin, NM, and DNA preparations from the mouse uterus (Metzger and Korach, 1990). As depicted in Figure 6 the chromatin, DNase-treated chromatin, and NM preparations exhibited similar binding characteristics; in all cases unlabeled ER demonstrated competitive binding for [^{3}H]ER as indicated by a decline in the percent maximal binding (Metzger and Korach, 1990). Thus the NM represents an enriched source of acceptor sites as it contained all of the binding present in the nucleus despite the removal of 90% and 85% of the cellular DNA and protein, respectively. Interestingly, the only preparation in which competition by ER for [^{3}H]ER did not occur was the pure DNA preparation, suggesting that the interaction of ER with uterine DNA was primarily nonspecific in nature and that DNA does not possess the selectivity necessary to function as a nuclear acceptor (Metzger and Korach, 1990; Schuchard, Rejman, McCormick,

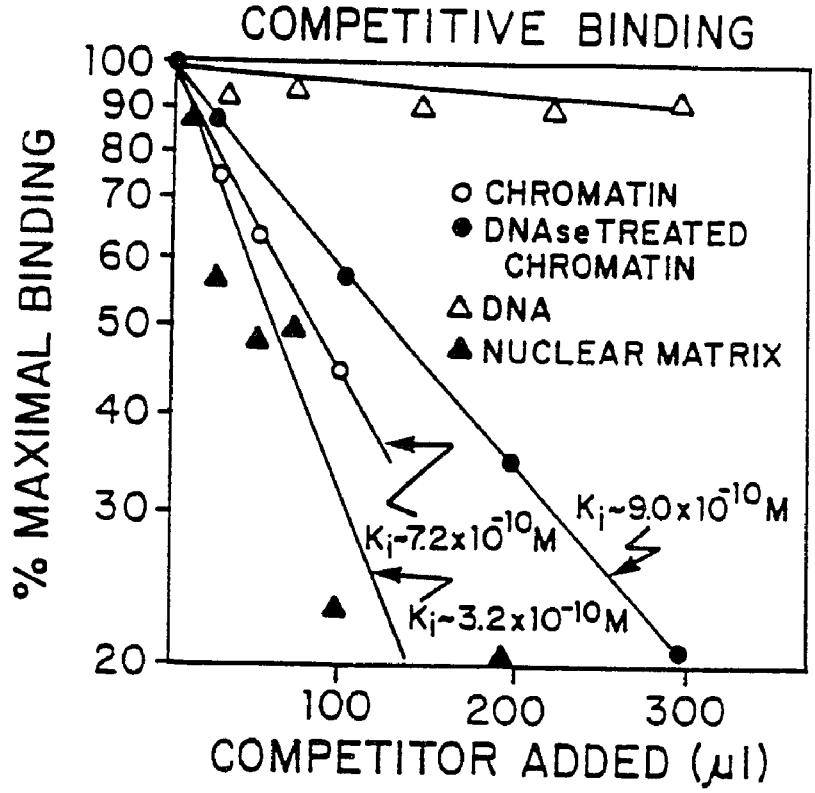


Figure 6. Competitive binding of [^{3}H]ER by increasing concentrations of ER using different sources of nuclear acceptor sites. Twenty-five microliters of [^{3}H]ER were incubated with increasing concentrations of ER and approximately one quarter uterine equivalent of nuclear acceptor site material. Reprinted from Metzger and Korach, 1990.

Gosse, Ruesink, and Spelsberg, 1991). The authors noted that the inhibition constant (K_i) was lowest for the NM preparation, possibly indicating the removal of nonspecific binding sites present in the other preparations (Metzger and Korach, 1990).

NM-receptor localization was thought to be physiologically significant because it only occurred in response to an appropriate hormonal stimulus. In one study, the correlation with hormonal status was demonstrated by the analysis of [^{3}H]E-ER binding to uterine NM prepared from estradiol and saline-treated mice in a cell-free assay system that did not permit exchange. The estradiol-treated mice had fewer acceptor sites available to bind [^{3}H]E-ER complexes due to occupancy by endogenous E-ER compared to saline-treated mice (Metzger and Korach, 1990). In addition, Barrack and colleagues (Alexander et al., 1987) reported that liver matrix prepared from ethinyl estradiol (EE)-treated rats exhibited specific [^{3}H]E binding whereas binding in saline-treated rats was barely detectable using an exchange assay system. This indicated that only the EE-treated rats had significant quantities of ER associated with their NM presumably due to exposure to EE *in vivo*. In addition to this exchange assay data, Barrack and colleagues demonstrated immunochemically that ER became associated with the NM following

exposure to estrogen *in vivo*. Monoclonal antibodies raised against the ER immunolocalized ER to NM prepared from the liver of EE-treated rats, but not untreated rats (Alexander et al., 1987). This study provided the first direct evidence that the association of ER with the NM was dependent on the presence of estradiol *in vivo*.

Numerous reports have described correlations between NM bound receptors and the stimulation of biological responses in a variety of peripheral target tissues for both androgens and estrogens (Barrack, 1987a; Barrack and Coffey, 1980, 1982; Barrack et al., 1977; Simmen, et al., 1984). For example, Barrack and Coffey (1980) reported a clear correlation between the specific binding of [^{3}H]E to liver NM and vitellogenin synthesis. Vitellogenin is synthesized by the liver of egg laying hens in response to E, and its synthesis was correlated with the association of ER with hen liver NM. Roosters, however, do not synthesize vitellogenin and had barely detectable levels of ER in liver NM. Interestingly, the administration of a pharmacological dose of E which results in the synthesis of vitellogenin mRNA, resulted in the association of ER with rooster liver NM (Deeley, Gordon, Burns, Mullinix, Binastein, and Goldberger, 1977). Furthermore, in the same study Barrack and Coffey (1980) reported that the rat ventral prostate NM contained specific binding

sites for AR that were rapidly lost (24 hr) following castration. This loss of NM-binding capacity correlated with the cessation of secretory function of the prostate. However, both NM sites and secretory function were restored following one hour of androgen replacement, indicating that the presence of AR in the rat prostate NM correlated with the stimulation of biological responses (Barrack and Coffey, 1980). Finally, physiological doses of E which induced uterine growth in immature female rats resulted in the association of ER with uterine NM, but not liver or lung NM. However, administration of pharmacological doses of E resulted in stimulation of liver biological response (angiotensin production) and the association of ER with liver NM (Barrack and Coffey, 1980).

It has been demonstrated that target tissues possess acceptor sites that are either absent or found only at low levels in nontarget tissues (Alexander et al., 1987; Barrack, 1987a; Metzger and Korach, 1990; Nelson et al., 1986). For example, higher levels of ER binding were exhibited in the NM of the uterus compared to nontarget tissue such as the spleen and lung (Agutter and Birchall, 1979; Metzger and Korach, 1990). Because DNA is invariant from cell to cell, what differs between target and nontarget tissues for a given animal may

be gene availability. The mechanisms governing availability may involve tissue-specific NMPs as discussed earlier (also see Fig. 3).

A Candidate Acceptor Protein for the Avian Oviduct Progesterone Receptor

The chromatin acceptor sites for progesterone receptors (PR) in the avian oviduct have been extensively studied by Spelsberg and colleagues (Hora, Horton, Toft, and Spelsberg, 1986; Horton, Landers, Subramaniam, Goldberger, Toyoda, Gosse, and Spelsberg, 1991; Landers and Spelsberg, 1992; Rories and Spelsberg, 1989; Ruh, Singh, Mak, and Callard, 1986; Spelsberg, 1982; Spelsberg, Littlefield, Seelke, Martin-Dani, Toyoda, Boyd-Leinen, Thrall, and Kon, 1983; Spelsberg, Rories, Rejman, Goldberger, Fink, Lau, Colvard, and Wiseman, 1989; Spelsberg, Ruh, Ruh, Goldberger, Horton, Hora, and Singh, 1988). Acceptor sites are composed of specific proteins bound to DNA with evidence for a sequence specific interaction (Rories and Spelsberg, 1989). Their research has shown that chromatin acceptor sites exhibit specific, high affinity interactions with PR both *in vivo* and *in vitro*, and represent the same class of acceptor sites localized on the nuclear matrix of the avian oviduct (Rejman, et al., 1991;

Schuchard, Subramaniam, et al., 1991). They have isolated an acceptor protein termed receptor binding factor-1 (RBF-1), in addition to two candidate acceptor proteins, RBF-2, and RBF-3 for PR (Horton, et al., 1991; Rejman, et al., 1991; Schuchard, Rejman, et al., 1991; Schuchard, Subramaniam, et al., 1991). RBF-1 is actually a complex consisting of 2 nonhomologous polypeptides termed RBF-1A and RBF-1B that comigrate during SDS-PAGE analysis (Lauber, Schuchard, Subramaniam, and Spelsberg, 1995). Most studies to date have been conducted on the RBF-1 complex which is a hydrophobic species- and tissue-specific nuclear protein complex that interacts with both PR and DNA and generates high affinity, saturable PR binding sites when reconstituted with genomic hen DNA (Schuchard, Rejman, et al., 1991; Schuchard, Subramanian, et al., 1991). Interestingly, the authors reported a direct correlation between PR binding capacity of a tissue and its concentration of RBF-1 (Schuchard, Subramanian, et al., 1991). In the absence of RBF-1, PR interactions with genomic hen DNA were linear and nonsaturable. The limited number of PR binding sites generated by the addition of RBF-1 to hen DNA indicated the involvement of a specific sequence of DNA in the establishment of these binding sites (Rejman, et al., 1991). Recently, a putative RBF-1 binding element (RBE) was

identified in the 5' domain of the avian c-*myc* gene (Lauber et al., 1995). Specific binding of the RBF-1 complex to the RBE was conferred by the polypeptide RBF-1B and preliminary evidence suggests RBF-1B expression was regulated by estrogen *in vivo* (Lauber at al., 1995; Sandhu and Spelsberg, 1995; Spelsberg, Lauber, Sandhu, Schuchard, and Subramaniam, 1995).

Immunoblots of nuclei, nuclear extracts and NM revealed that all nuclear RBF-1 in the chick oviduct was associated with the NM (Schuchard, Subramanian, et al., 1991). In addition, NM-associated RBF-1 exhibited a tissue-specificity that mimicked that of nuclear RBF-1. Furthermore, Southwestern blotting experiments revealed that ^{32}P-labeled NM DNA prepared from chick oviduct interacted specifically with RBF-1 (Schuchard, Subramanian, et al., 1991). Interestingly, PREs were not detected in the NM DNA, however, it is possible that the PRE, which may be located upstream of the RBF-1 binding site, was destroyed by the extensive DNase digestion utilized in the preparation of NM. In addition, despite previous reports that the ovalbumin gene was localized on the NM in the avian oviduct (Ciejek, et al., 1983) the authors were unable to hybridize ovalbumin genomic DNA to the NM DNA. Again, the extensive DNase digestion, which resulted in shorter NM DNA fragments than reported by other

investigators, may be the reason for this discrepancy (Schuchard, Subramaniam, et al., 1991). Recent immunohistochemical studies have colocalized RBF-1 and PR in nuclei of the chick oviduct (Zhaung, Landers, Schuchard, Syvala, Gosse, Ruesink, Spelsberg, and Tuohima, 1993). In addition, Western blotting studies have illustrated that RBF-1 was upregulated in the presence of estradiol, and qualitative changes occurred in response to progesterone treatment (doublet formation; Zhuang, et al., 1993). An RBF-1-like protein of similar molecular weight (15 kDa) was observed in the rat uterus, by both immunohistochemical and western blotting procedures (Zhaung et al., 1993). The relationship between the rat and avian RBF-1 protein is unclear at present, but its presence in both species suggests evolutionary conservation of this protein. Acceptor proteins of similar molecular weight have been identified for estrogen, androgen and thyroid receptors (Burnside, Darling and Chin, 1990; Lazar and Berrodin, 1990; Murray and Towle, 1989; Rennie, Bowden, Bruchovsky, Frenette, Foekens, and Chen, 1987; Ross and Ruh, 1984; Ruh, Burroughs, and Ruh, 1995; Ruh and Ruh, 1988; Singh, Ruh and Ruh, 1986; Singh, Ruh, Butler and Ruh, 1986). Together with reports of the presence of RBF-1 in several glucocorticoid-responsive/ progesterone-unresponsive tissues, this suggests the presence of a

conserved family of acceptors for SR (Zhaung, et al., 1993). It is likely that these NM-associated acceptor proteins play a common role in tissue-specific gene regulation in hormonally responsive tissue for all members of the nuclear receptor superfamily; research in this area is ongoing.

Relevance of Estrogen Receptor-Nuclear Matrix Interactions in Neural Tissue

The colocalization of transcriptionally active genes, transcription factors, SR, and SR acceptor proteins on the NM makes the NM a strong candidate for the functionally significant nuclear binding site for the steroidal regulation of hormone-responsive genes. Remarkably, with the single exception of a study that examined corticosteroid binding in the hippocampus (van Steensel, et al., 1991), all studies of SR interaction with NM have utilized peripheral target tissues or cell lines.

Given the presence of hormonally regulated NMPs in peripheral targets and cell lines and their postulated role in tissue-specific gene expression, characterization of neural SR-NM binding potentially could provide insights into the mechanisms involved in the

development and expression of sexual dimorphisms in hormone sensitivity and regional variability in gene regulation. For example, the mechanism that determines target tissue sensitivity presumably due to the association of hormone-regulated genes with the NM may also regulate sexual dimorphisms in hormone sensitivity. Hormonal regulation of NMP constituents may determine the extent to which specific genes are available for transcription in males and females, based upon their association with the NM. If this is the case it would become possible to elucidate the mechanism by which hormones sexually differentiate neural tissue, opening up another level of analysis for the biobehavioral actions of steroid hormones. Some examples of sexual dimorphisms that may arise from such a NM-mediated mechanism are described below.

Sexual Dimorphisms in Hormone Sensitivity

Male and female mice differ in responsiveness to the aggression-promoting properties of testosterone (T) and estradiol (E). When gonadectomized during adulthood, both will fight in response to T. However males require a shorter period of exposure than females, 2-3 versus 22 days, respectively (reviewed in Simon, Lu,

McKenna, Chen and Clifford, 1993; Simon, McKenna, Lu, and Cologer-Clifford, 1996). Neonatal exposure of females to T eliminates this sex difference, although exposure must occur within a restricted period (reviewed in Gandelman, 1980). In regard to E, gonadectomized males are highly responsive to the aggression-promoting property of this hormone while females are completely insensitive (Simon et al., 1993; 1996; Simon and Gandelman, 1978; Simon, Whalen and Tate, 1985). However, females will display aggression if they are exposed to E or an aromatizable androgen early in neonatal development (Klein and Simon, 1991; Simon et al., 1993; Simon and Whalen, 1987; Suarez, Cologer-Clifford, and Simon, 1992).

The sex difference in the response to E extends to other systems as well. As previously described, one marker of E action is the induction of PR in the ventromedial nucleus (VMN) of the hypothalamus, the appearance of which is highly correlated with the expression of lordosis, a female-typical behavior (Etgen, 1984). Following exposure to E, gonadectomized female rats had a significantly higher concentration of PR within the VMN than gonadectomized male rats (Brown, Clark and MacLusky, 1987; Parsons, Rainbow and McEwen, 1984; Rainbow, Parsons and McEwen, 1982). Perinatal exposure of female rats to E or T resulted in male-

typical E-induced PR concentrations and lordosis profiles in adulthood (Parsons et al., 1984). Complementing these observations are results showing that prenatal exposure of male rats to an aromatase inhibitor, which blocks the metabolic conversion of T to E, resulted in female-typical E-induced PR concentrations and lordosis profiles (Parsons et al., 1984).

The mechanism that underlies the establishment of these sexual dimorphisms in hormone sensitivity is unknown. It is plausible that the hormonal environments in males and females during the pre- and perinatal periods may regulate the availability of genes via their association with the NM (possibly due to hormonal regulation of NMPs) and thereby play a role in the establishment of the sexual dimorphism in hormone sensitivity apparent in adulthood. The ability to reverse these sex-typical biobehavioral responses by prenatal exposure to, or deprivation of E represents an interesting paradigm to assess the role of the NM and NMPs in the establishment of sexual dimorphisms in hormone sensitivity.

Furthermore, the seemingly hardwired effects of prenatal hormonal environment may be overcome, in some instances, by prolonged exposure to or deprivation of hormones in adulthood. For example, long-term T exposure enabled female mice to exhibit male-

typical aggression (Simon et al., 1993; 1996). Complementing this observation are data concerning the effects of long term hormone deprivation on the ability of E to induce PR and activate lordosis in female rats. Long term ovariectomy (OVX; 4-5 weeks) that did not affect ER concentrations, decreased the ability of E to induce PR in the mediobasal hypothalamus of the rat brain and reduced the ability of E + P treatment to activate lordosis (Barley, Ginsburg, MacLusky, Morris, and Thomas, 1977; Clark, MacLusky, Parsons, and Naftolin, 1981; Delville and Blaustein, 1989). Rats implanted with capsules of E at the time of OVX did not exhibit this loss in estrogen sensitivity, indicating that E deprivation altered the ability of E to activate the PR gene (Delville and Blaustein, 1989). It is plausible that prolonged T exposure, in the case of aggression, and E deprivation in the case of PR induction, resulted in enhanced and reduced access, respectively, to the pertinent genes due to a change in chromatin structure driven by changes in NMP content.

In keeping with the concept of hormonal regulation of gene availability, and directly relevant to hormonal effects in the neural tissue, Ventanas and coworkers (Ventanas, Garcia, Lopez-Bote, Lopez, and Burgos, 1990) reported that *in vitro* androgen receptor (AR) binding to deproteinized hypothalamic chromatin in mice was

positively correlated with neonatal androgen exposure. Although this study did not utilize the NM per se, the NM represents the active constituent of deproteinized chromatin and exhibited binding characteristics similar to both chromatin and DNase-treated chromatin (see earlier discussion of NM as acceptor site; Barrack, 1987a; Hora, et al., 1986; Metzger and Korach, 1990). One consequence of male sexual differentiation is the capacity of the hypothalamus for binding high concentrations of AR. In the study by Ventanas and coworkers, males exhibited significantly higher levels of AR binding than females. However, when females were administered testosterone proprionate in the first 24 hours of life, they had significantly greater levels of AR binding than the untreated females. Neonatal exposure to androgens may have resulted in the establishment of a "male-typical" chromatin configuration that rendered more acceptor sites accessible in the untreated males.

Rationale for the Present Study

As described earlier, there are several examples of hormonally-modulated NMPs in breast, prostate, seminal vesicle, and

cell lines. In addition, there is evidence that the PR acceptor protein characterized by Spelsberg and colleagues, RBF-1, is hormonally regulated. In light of increasing evidence of the role of NMPs in cell-, tissue-, transformation-, and differentiation-state- dependent gene expression, it is plausible that sexual dimorphisms in steroid sensitivity could be regulated by a similar mechanism. However, NM-gonadal steroid receptor interactions have never been examined in neural tissue. To this end, the present study was conducted to demonstrate that ER interacts with NM of limbic origin as has been documented in several peripheral estrogen targets. The limbic system was selected as the target because it subsumes the regions known to play a role in the regulation of estrogen- and androgen-inducible sex-typical biobehavioral responses in rodents (Barfield and Chen, 1977; Lisk, 1962; Owen, Peters and Bronson, 1974; Sheridan, 1978) and it is rich in E-concentrating cells (Lu, unpublished data, Pfaff, 1968; Pfaff and Keiner, 1973: Stumpf and Sar, 1974). ER-NM interactions were characterized both biochemically and immunochemically, establishing a level of analysis for the physiological actions of steroid hormones previously unavailable in neural tissue.

CHAPTER TWO: GENERAL METHODS

Chemicals. DNase I was obtained from Boehringer-Mannheim and phenylmethylsulphonylfluoride (PMSF) from Sigma. Hormones were purchased from Steraloids and all other reagents were purchased from Sigma. [^{3}H]-labeled estradiol was purchased from New England Nuclear.

Animals and surgery. Female CD-1 mice (60-70 days of age) purchased from Charles River Breeding Farm (Wilmington, MA) were group housed and given lab chow and water ad libitum. Females were ovariectomized via bilateral incisions on the flanks posterior to the last rib under Nembutal (Pentobarbital) anesthesia 1-2 weeks prior to use. All maintenance procedures were in compliance with Federal guidelines for animal care.

Treatments. Females received 20 μg estradiol benzoate (EB) administered subcutaneously in 0.02-cc corn oil prior to sacrifice by cervical dislocation. The interval between EB injection and sacrifice is noted for each experiment. This dose was selected based on its

ability to render ovariectomized female mice sexually receptive (unpublished observations).

Nuclear isolation. Brains were rapidly removed and blocked on ice. Dissection of the limbic area included the hypothalamus extending from the mammilary bodies to the optic chiasm, the preoptic area, and septum. Subsequent steps were performed at 0° C and all buffers contained 0.1 mM PMSF added from a 0.1 M stock in ethanol immediately before use. Sections from 8 mice were homogenized in 7 ml ice-cold 0.25 M STM (0.25 M sucrose, 10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) with 10 4 second bursts of a hand held mechanical homogenizer (Biospec M100) followed by a 3 ml wash. The homogenate and wash were combined and centrifuged in a fixed angle rotor at 800 x g for 15 min. in an IEC Centra 7R centrifuge. The supernatant was decanted and the pellet resuspended in 10 ml 0.25 M STM buffer and the above centrifugation repeated. The supernatant was decanted and the pellet resuspended in 7 ml 2 M STM buffer (2 M sucrose, 10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) and spun for 60 min. at 25,000 x g using a Beckman SW 50.1 rotor in a Beckman L8-70 Ultracentrifuge. The floating debris and supernatant were discarded and the tubes inverted and wiped dry. The pellets

were resuspended in 0.25 M STM and centrifuged for 15 min. at 800 x g. This wash was repeated once more and the pellet resuspended in 0.25 M STM and an aliquot of nuclei was taken for DNA quantitation using the Hoechst reagent. This nuclear preparation was subjected to exchange assay and immunochemical analysis where indicated.

Nuclear matrix isolation. The NM was isolated following a modification of the methods of Barrack (1983) and Metzger and Korach (1990). Briefly, nuclei were extracted sequentially with Triton X-100 (1% in 0.25 M STM), DNase I (78.7 U/ml in TM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4), low magnesium buffer (10 mM Tris-HCl, 0.2 mM $MgCl_2$, pH 7.4) to which 1.1 M TEK (10 mM Tris-HCl, 1.5 mM EDTA 1.1 M KCl, pH 7.6) was added to bring the final concentration of KCl to 1 M. The NM was resuspended in 0.6 M TEK (10 mM Tris-HCl, 1.5 mM EDTA, 0.6 M KCl, pH 7.6) and used immediately. The spherical integrity of the NM was assessed via phase contrast microscopy at each step of the NM protocol.

Exchange assay. Incubations were conducted in a total volume of 500 µl in 12 x 75 mm glass tubes that were pretreated with 0.1 % BSA

prior to use. The incubates consisted of NM in the presence of a range of concentrations of [^{3}H]E as noted for each experiment ([^{3}H]E; New England Nuclear, Boston, MA, specific activity 126 - 142 Ci/mmol). NM was incubated alone (to measure total binding) or in the presence of a 1000 molar excess of unlabeled E (to measure nonspecific binding). Incubations were conducted at 37° C for 45 min. in a shaking water bath (Precision) followed by a 5 min. incubation at 0°C. The NM was pelleted and washed in 2 ml TEK (0.6M) four times by centrifugation (10-15 min. at 800 x g). Following the final wash the walls of the tubes were wiped with ethanol and dried with cotton swabs. The pellets were extracted with 1 ml absolute ethanol with periodic vortexing and transferred to scintillation vials followed by the addition of 5 ml of Ecoscint O. Samples were counted in a Beckman LS-8100 liquid scintillation counter. Efficiency was determined by the external standard channels ratio method. Specifically bound hormone was calculated by subtracting nonspecific from total binding and was expressed as femtomoles of receptor bound per 100 μg DNA present in the nuclear pellet before DNase digestion (DNA nuclear equivalent). Saturation curves were analyzed by the method of Scatchard (1949).

DNA Determination. DNA was quantified by the use of the Hoechst reagent (H33258). Calf thymus DNA (Sigma) was used as a standard. Due to the low concentration of DNA present in the preparation, a microplate assay protocol was developed based on the method of Labarca and Paigen (1980). DNA values reported in the experiments represent amount of DNA present in the preparation prior to NM preparation.

CHAPTER THREE: EXPERIMENTS

Preliminary Study:

ER-NM Interactions in a Whole Brain Preparation

To minimize the number of mice used in the development of the NM isolation protocol, preliminary studies utilized whole brain from EB-treated female mice to obtain a high yield of DNA. The final version of the NM protocol is detailed in the general methods section and consists of a modification of the protocols of Barrack (1983) and Metzger and Korach (1990).

Methods

Females received injections of 20 μg EB 48 and 24 hr prior to sacrifice. Because whole brain preparations included a large number of regions that do not contain ER (Stumpf and Sar, 1975), neuronal cells were separated from glial cells by the method of Thompson (1973) to maximize the percentage of target tissue in the sample. All steps were carried out at 4° C. Briefly, 4 whole brains were homogenized in 20% (w/v) 2 M sucrose/1 mM $MgCl_2$ and centrifuged for 30 min. at 64,000 g in a swinging bucket rotor (Beckman SW 50.1). The nuclear pellet was resuspended in 3.6 ml 2.4 M sucrose/1 mM

$MgCl_2$ and overlayed with 1.4 ml of 1.8 M sucrose/1 mM $MgCl_2$ and centrifuged in a swinging bucket rotor for 30 min. at 85,000 g. The 1.8 M sucrose overlay was discarded. The interface and 2.4 M sucrose solution containing the neuronal fraction and the pellet containing the glial fraction were transferred to separate 15 ml Corex tubes. Microscopic examination of neuronal and glial fractions revealed little cross contamination of neuronal and glial cell populations. The neuronal fraction was diluted 2 fold with 0.32 M sucrose/1 mM $MgCl_2$, mixed with gentle vortexing, and then spun at 2,000 g for 15 min. in a Sorvall RC5B in a SS34 rotor. The neuronal pellet was used in the preparation of NM as described above and was assayed with 0.5 - 25 nM [^{3}H]E.

Results and Discussion

NM prepared from whole brains of EB-treated females exhibited specific ER binding when assayed with 0.5 - 25 nM [^{3}H]E (see Figures 7A and B for saturation and Scatchard plots), however, nonspecific [^{3}H]E binding interactions were problematic at high concentrations. Interestingly, glial fractions also exhibited specific binding (data not shown). These results suggested that analysis of ER-NM interactions in neural preparations was plausible and the use of circumscribed brain

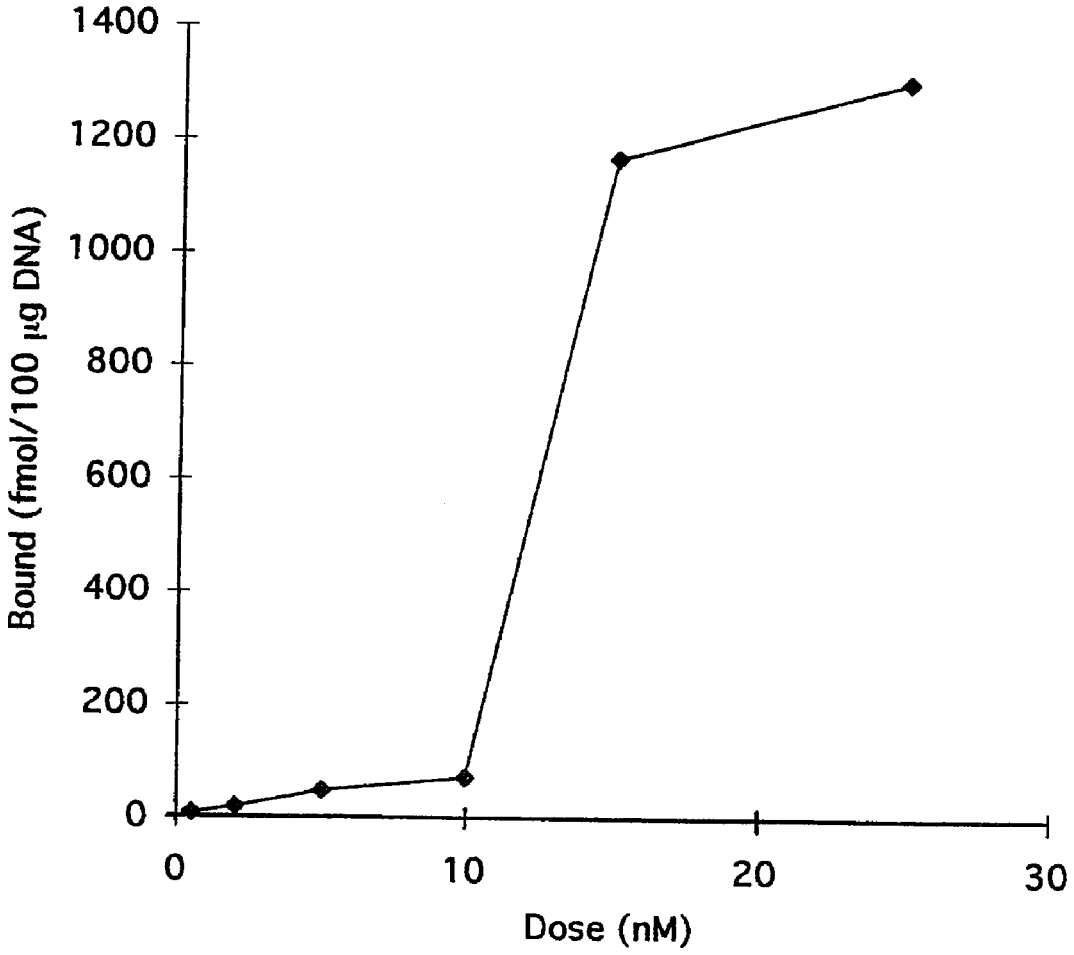


Figure 7A. Specific binding of estradiol to whole brain nuclear matrix of estrogen-treated female mice. Nuclear matrix was isolated from whole brains of estrogen-treated females. Estradiol binding was quantitated by an *in vitro* exchange assay as described in the text. Specific binding was quantitated utilizing 0.5-25 nM [^{3}H]E. Each point represents the mean of 3-4 assays conducted with internal triplicates.

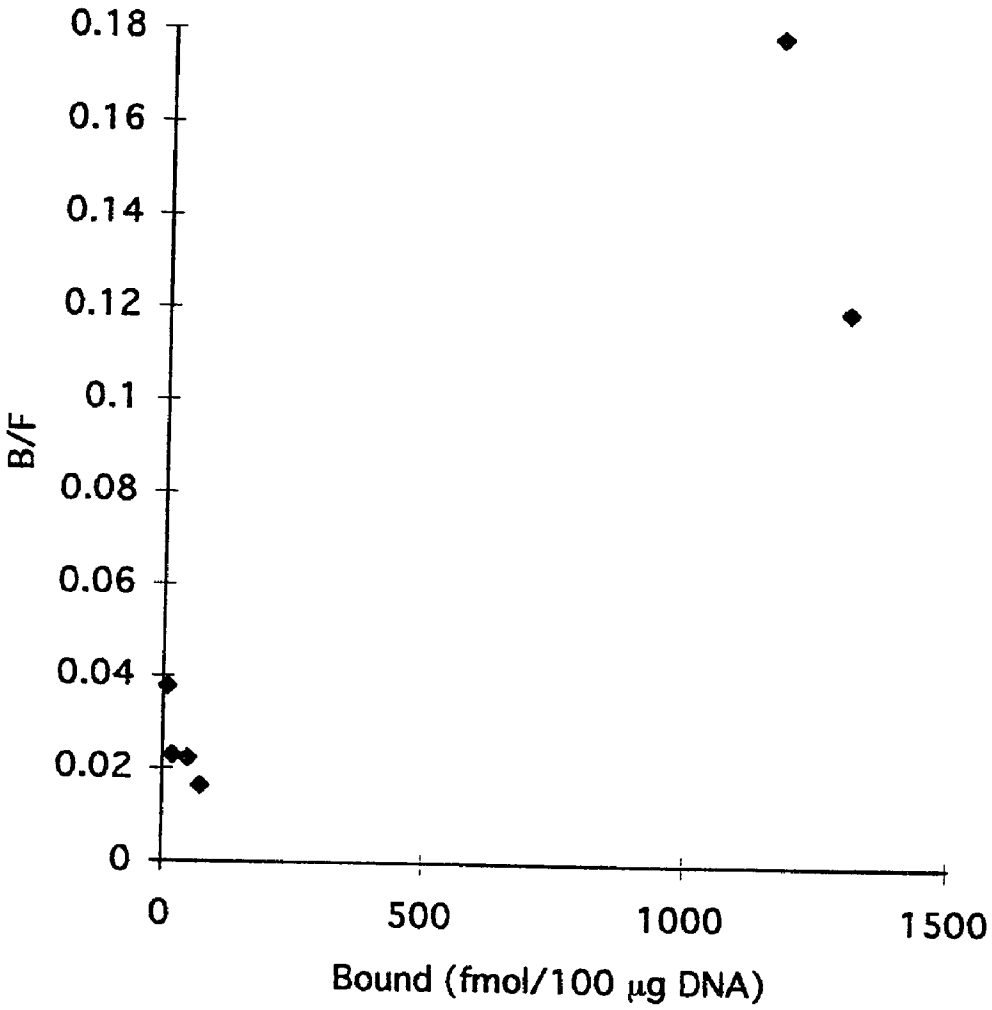


Figure 7B. Scatchard analysis of the data in Figure 7A.

regions enriched in ER would likely generate meaningful data. To this end, subsequent experiments utilized the limbic region of the brain as outlined in the general methods. Because this region is rich in ER (Pfaff, 1968; Pfaff and Keiner, 1973; Stumpf and Sar, 1974), neuronal/glial separation was not carried out in subsequent experiments.

Saturation Analysis of Limbic Region: I

Methods

Females received injections of 20 μg EB, 48 and 24 hr prior to sacrifice. NM was prepared from 15 mice and aliquots containing an average of 13 μg DNA were subjected to exchange assay described above utilizing 2-16 nM [^{3}H]E.

Results and Discussion

Saturation and Scatchard analyses of the data are depicted in Figures 8A and 8B, respectively. Based on the saturation curve, there seemed to be at least two binding components present. The existence of multiple binding sites was confirmed by the curvilinear nature of

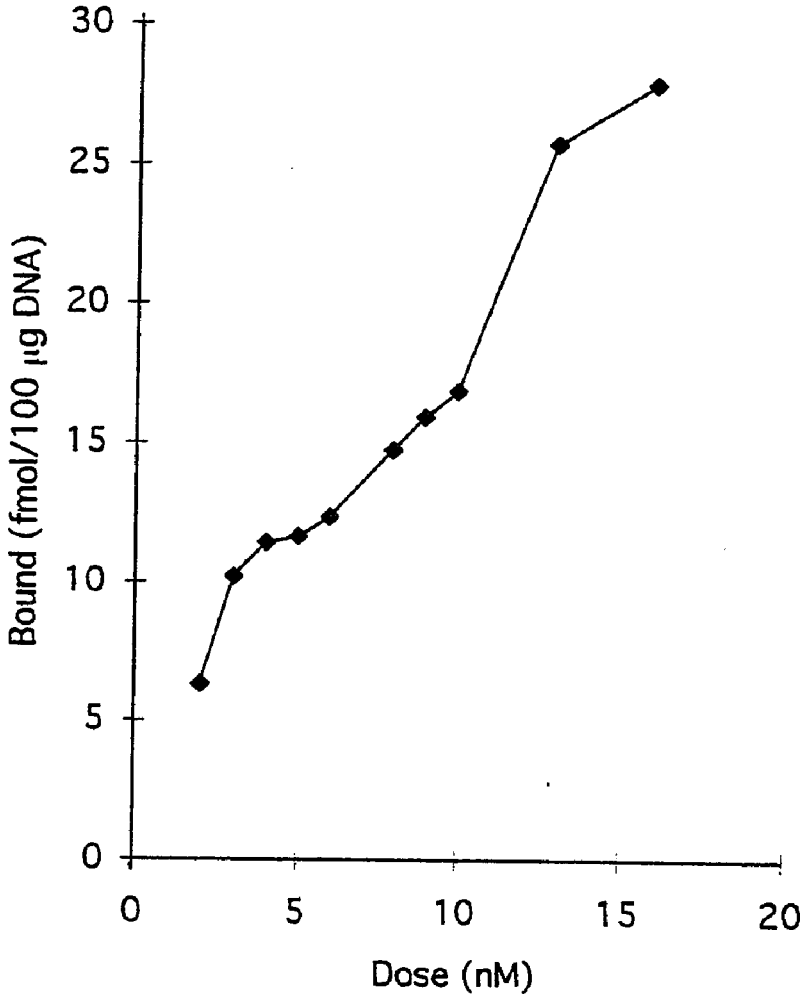


Figure 8A. Specific binding of estradiol to limbic nuclear matrix of estrogen-treated female mice. Nuclear matrix was isolated from limbic tissue from estrogen-treated females. Estradiol binding was quantitated by an *in vitro* exchange assay as described in the text. Each assay tube contained 13 µg DNA nuclear equivalent. Specific binding was quantitated utilizing 2-16 nM [^{3}H]E. Each point represents the mean of 3-4 assays conducted with internal triplicates.

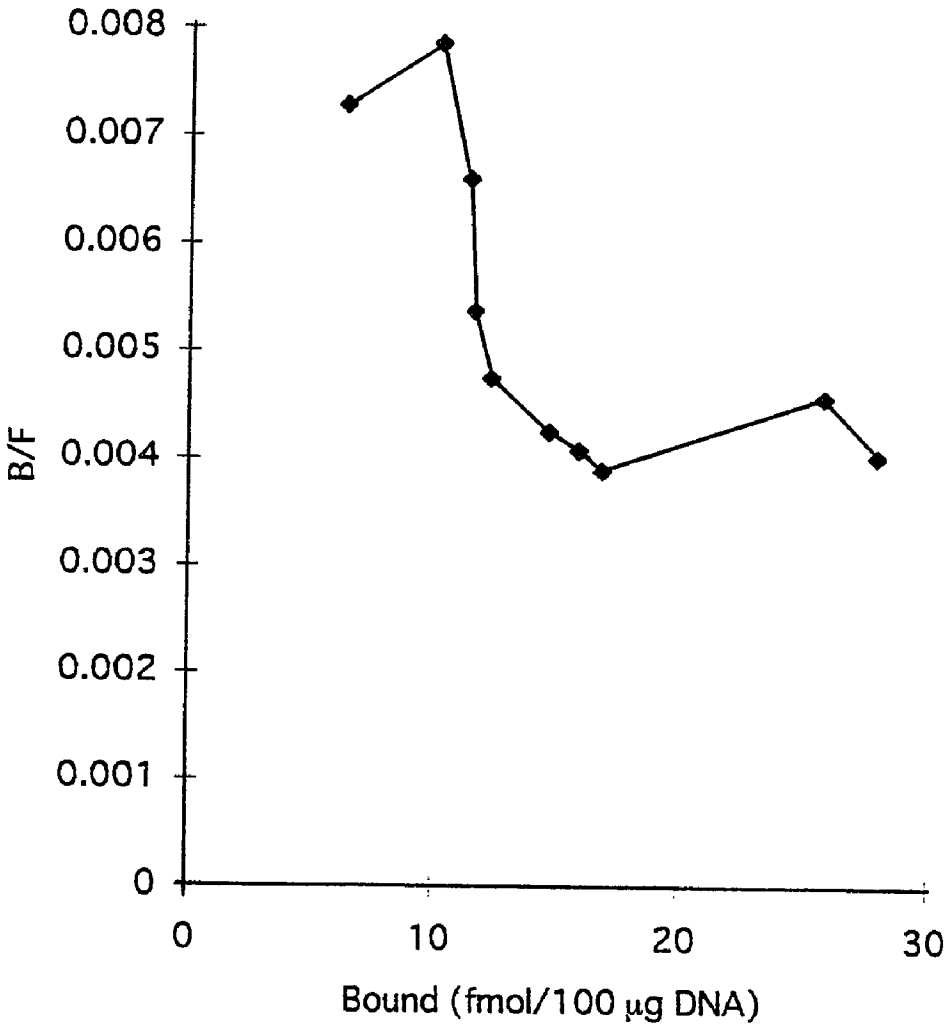


Figure 8B. Scatchard plot of the data in Figure 8A.

the Scatchard plot. Curve fitting analysis (Enzfitter) was carried out for doses in the range of 2-10 nM [^{3}H]E because this range likely contained the first two binding components and seemed amenable to analysis. The curve fitting algorithm identified 2 sites with the following characteristics: 1) $K_d = 0.135 \times 10^{-9}$ M with $B_{max} = 6.4$ fmol/100 µg DNA; 2) $K_d = 3.53 \times 10^{-1}$ with $B_{max} = 45.67$ fmol/100 µg DNA.

The high affinity site exhibited K_d and B_{max} that were basically consistent with that reported for ER-NM interactions in peripheral estrogen target tissues such as rat and mouse uterus, avian liver and human prostate (K_d = 0.38 - 2.6 nM; Barrack and Coffey, 1982; Barrack, 1987a; Markaverich and Clark, 1979; Metzger and Korach, 1990; Metzger, et al., 1991; Simmen, et al., 1984). It was suggested that the second binding component may represent the type II estrogen binding site (type II EBS; Densmore, personal communication). The type II EBS is a low affinity estrogen binder present in significant excess over ER in a number of estrogen-responsive tissues including rat uterus, avian liver and oviduct, and the rat and human prostate (Clark, et al., 1982; Ekman, Barrack, Greene, Jensen, and Walsh, 1983; Markaverich and Clark, 1979; Markaverich, Williams, Upchurch, and Clark, 1981; Simmen, Means,

and Clark, 1984). However, the possibility that the second site represented a procedural artifact could not be ruled out based on previous experience with problematic nonspecific binding interactions at high [^{3}H]E doses in whole brain preparations described above. The extremely low affinity of the second site, together with the aberrant shapes of the saturation curve and resulting Scatchard plot raised concerns as to the ability of the curve fitting algorithm to accurately resolve the two binding components. This called into question the precision of the estimation of the parameters of the high affinity binding component.

A serious constraint on the interpretation of data was the inability to detect specific binding below a dose of 2 nM [^{3}H]E. This dose of [^{3}H]E represents a value 20 times the calculated K_d. Therefore, the calculated K_d fell on a portion of the saturation curve that represented an extrapolation of the data rather than the data itself. All subsequent experiments were conducted with a 3.4-fold higher concentration of DNA per assay tube in an attempt to quantitate binding interactions utilizing doses in the range of K_d. In addition, to ensure maximal signal detection in subsequent experiments, the following analysis of the timecourse of appearance of estrogen binding sites on NM following sc. injections of EB in oil was conducted.

Timecourse of Appearance of Estrogen Binding Sites on NM

Methods

Females received injections of EB 2, 4, 8, 12, and 24 hr prior to sacrifice. NM was prepared from 8 females per group and aliquots containing an average of 44 μg DNA were subjected to exchange assay utilizing 5 nM [^{3}H]E.

Results and Discussion

As depicted in Figure 9, ER-NM binding was maximal at 12 hours post EB injection, therefore this time point was utilized for subsequent experiments.

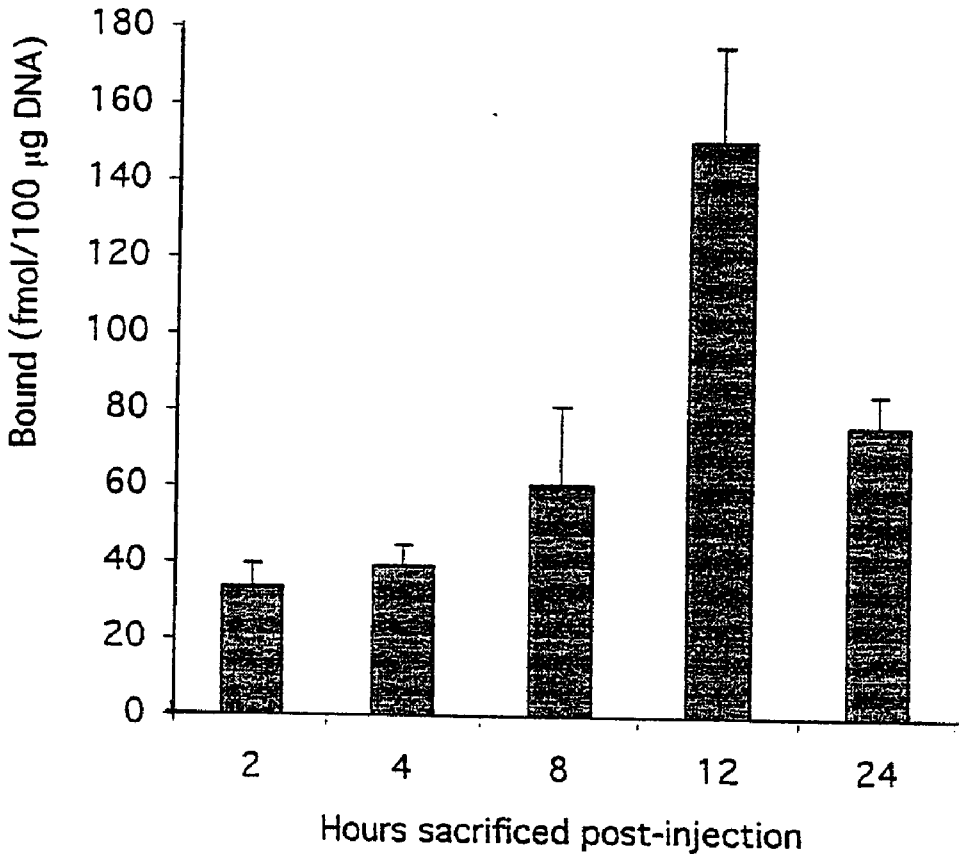


Figure 9. Timecourse of appearance of [^{3}H]E binding on limbic nuclear matrix in the brain of estrogen-treated female mice. Nuclear matrix from estrogen-treated females (44 µg DNA nuclear equivalent) was incubated with 5 nM [^{3}H]E 2, 4, 8, 12, and 24 hours post injection. Each point represents the mean of internal triplicates + SEM.

Saturation Analysis of Limbic Region: II

Methods

Based on the preceding studies, females received injections of EB 12 hours prior to sacrifice. In addition, some females received oil vehicle injections to demonstrate that ER association with NM was dependent upon estrogenic stimulation. NM was prepared from 15 mice per experiment and aliquots containing an average of 44 μg DNA were subjected to exchange assay utilizing a range of 0.2-5 nM [^{3}H]E.

Results and Discussion

Saturation and Scatchard analyses for EB-treated mice are depicted in Figure 10A and 10B. The shape of the saturation curve and the Scatchard plot again revealed the presence of multiple binding sites. The range of 0.2 - 1.2 nM [^{3}H]Ewas utilized for the Scatchard analysis. The NM of EB-treated mice exhibited specific, high affinity [^{3}H]Ebinding: K_d =1.15 X 10 $^{-9}$ M and B_{max} = 4.38 fmol/100 μg DNA. Analysis of [^{3}H]E binding in nuclear samples from EB-treated females indicated that approximately 80% of the nuclear binding remained associated with the NM (data not shown). As in the previous study,

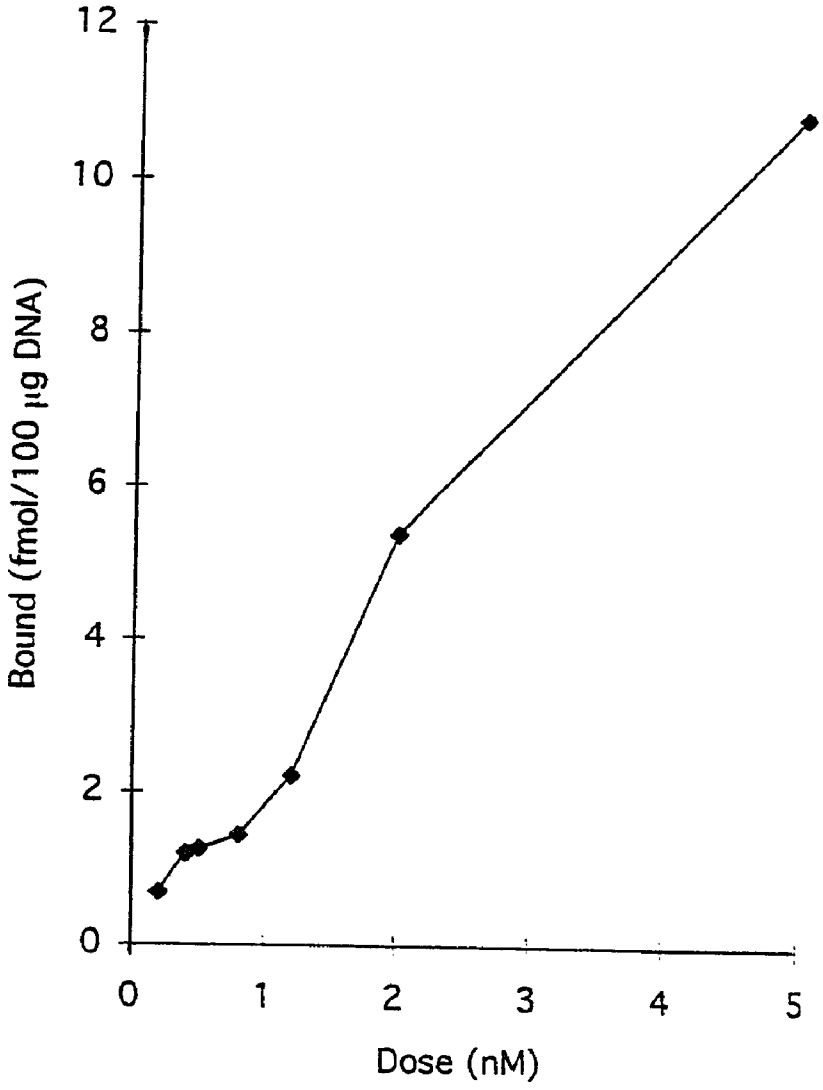


Figure 10A. Specific binding of estradiol to limbic nuclear matrix of estrogen-treated female mice. Nuclear matrix was isolated from limbic tissue from estrogen-treated females. Estradiol binding was quantitated by an *in vitro* exchange assay as described in the text. Each assay tube contained 44 µg DNA nuclear equivalent. Specific binding was quantitated utilizing 0.2-5 nM [^{3}H]E. Each point represents the mean of 3-4 assays conducted with internal triplicates.

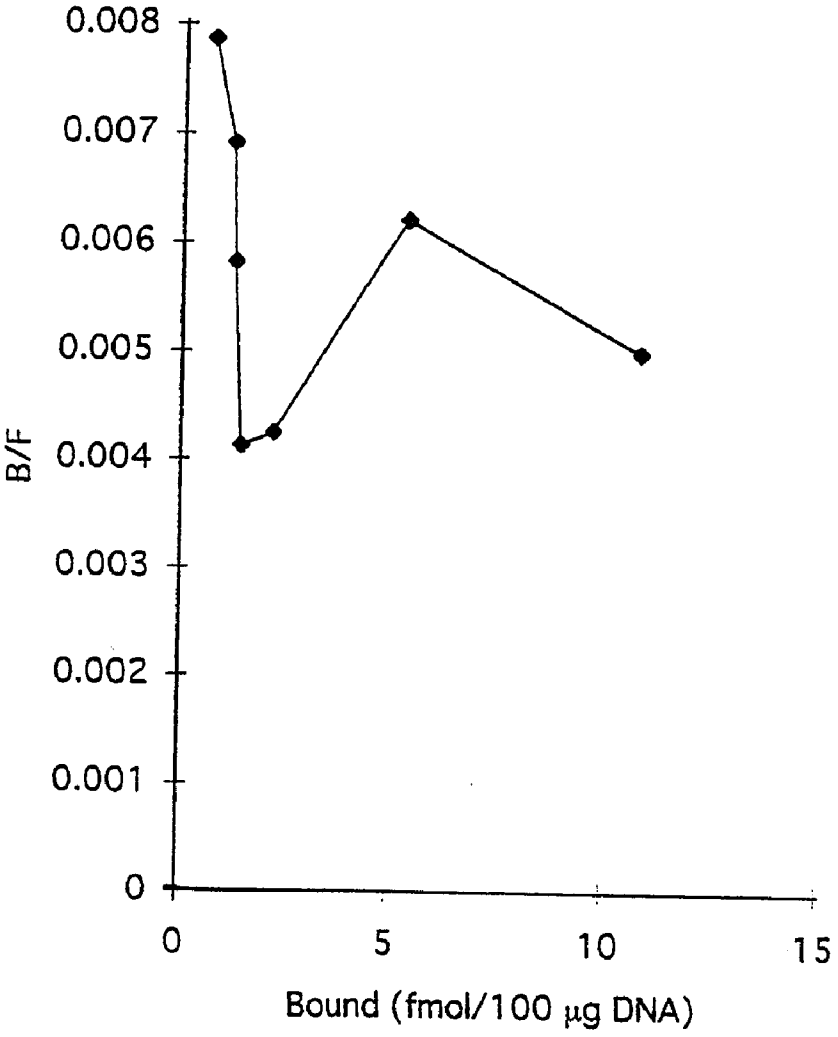


Figure 10B Scatchard plot of the data in Figure 10A.

the K_d was consistent with that described for ER-NM interactions in peripheral tissues (K_d = 0.38 - 2.6 nM; Barrack and Coffey, 1982; Barrack, 1987a; Markaverich and Clark, 1979; Metzger and Korach, 1990; Metzger, et al., 1991; Simmen et al., 1984). However, it is an order of magnitude higher than the K_d obtained from the preceding experiment. Due to the expanded dose range utilized in this assay, the present data may represent a more reliable estimate of the binding parameters. However, because the contributions of the second binding component could not be partitioned out, the accuracy of the determination of K_d is questionable. Evidence to this effect comes from an inspection of the saturation curve which indicates that half maximal saturation occurred at about 0.6 nM for the first binding component rather than the value of 1.15 nM generated by the Scatchard analysis. B_{max} is similar in the present and preceding experiments, 4.38 fmol/100 μg DNA and 6.4 fmol/100 μg DNA, respectively. Again, these data must be interpreted cautiously due to the inability to partition out the contributions of the second binding component. The hyperbolic nature of the second binding component detected at higher doses of [^{3}H]Eprecluded analysis by the method of Scatchard, and was similar in shape to the type II EBS curve reported in rat uterine preparations (Markaverich and Clark,

1979). The saturation and Scatchard plots from NM of oil-treated mice are depicted in Figure 11A and 11B. Oil-treated females exhibited a low level of [^{3}H]E binding consistent with the lack of estrogen *in vivo*. ER-NM interactions in oil-treated mice were undetectable below the 0.5 nM dose of [^{3}H]E and remained extremely low below the dose of 1.2 nM [^{3}H]E. In a manner similar to EB-treated females, there was a large increase in bound [^{3}H]E at the 1.2 nM dose that likely represents the presence of the second binding component. Not enough data was generated to conduct a Scatchard analysis.

There are numerous accounts of interference by type II EBS in the quantitation of nuclear ER binding (Clark, et al., 1982; Ekman, Barrack, Greene, Jensen, and Walsh, 1983; Markaverich and Clark, 1979; Markaverich, Williams, Upchurch, and Clark, 1981; Simmen, et al., 1984). Accurate quantitation of nuclear ER can be carried out if reducing agents such as dithiothreitol (DTT), which inhibit the binding of [^{3}H]E to these low affinity sites, are added to assay tubes prior to exchange (Markaverich, et al., 1981). However, this requires knowledge concerning the presence of these sites apriori. When this is not this case, extrapolation of the steepest component of the Scatchard curve to the x-axis has been used to estimate B_{max} (Ekman, et al., 1983;

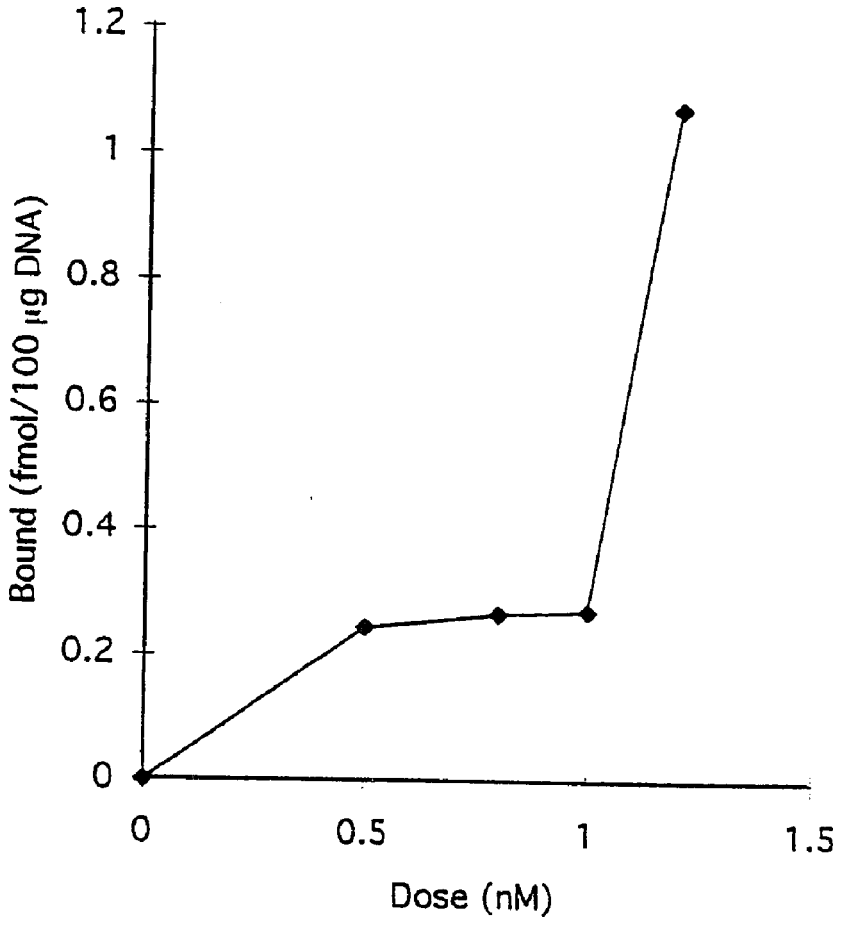


Figure 11A. Specific binding of estradiol to limbic nuclear matrix of oil-treated female mice. Nuclear matrix was isolated from limbic tissue from oil-treated females. Estradiol binding was quantitated by an *in vitro* exchange assay as described in the text. Each assay tube contained 44 µg DNA nuclear equivalent. Specific binding was quantitated utilizing 0.2-5 nM [^{3}H]E. Each point represents the mean of 3-4 assays conducted with internal triplicates.

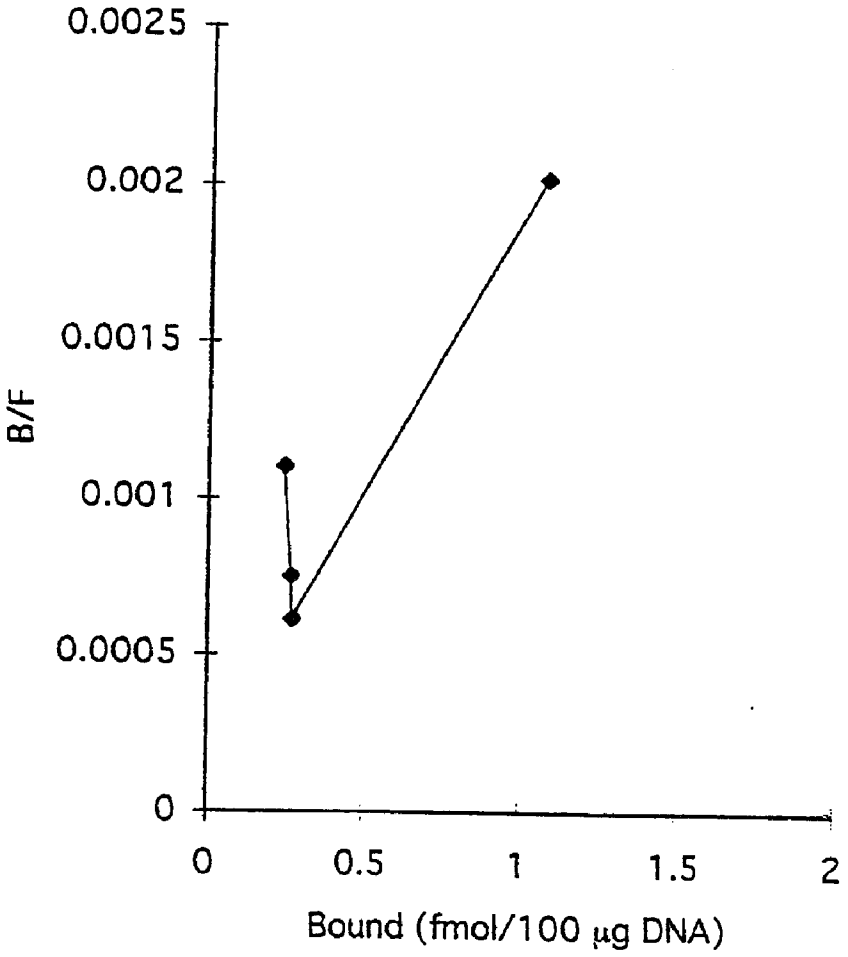


Figure 11B. Scatchard plot of the data in Figure 11A.

Markaverich and Clark, 1979). In the present study use of this method for the estimation of B_{max} relied on a restricted portion of the dose range, 0.2 - 1.2 nM. In keeping with this approach, Barrack and coworkers (Ekman, et al., 1983) noted the importance of an adequate number of concentrations in the range of 0.01-1.0 nM [^{3}H]E to accurately quantitate high affinity nuclear [^{3}H] Ebinding sites in human prostate where type II EBS are present.

While it would have been possible to again increase the amount of DNA per assay tube in an attempt to collect binding data in the dose range between 0 and 1.0 nM, in addition to implementing procedures to preclude interference by type II EBS, this would have meant a substantial increase in the number of mice used in the study, which was not feasible for a number of reasons. In addition, it would not have addressed a limitation inherent in working with neural tissue, cellular heterogeneity. Although the limbic region represents an enriched source of ER, not every cell in this region contains ER (Lu, unpublished data; Pfaff, 1968; Pfaff and Keiner, 1973; Stumpf and Sar, 1974). The presence of nontarget cells increases the source of nonspecific binding interactions and dilutes the signal strength.

For these reasons a change in methodology was sought that would enable the detection of NM-associated ER without interference from

type II EBS, without dilution of signal strength by nonspecific interactions, and that would not involve the use of large numbers of mice. Previously, ER-NM interactions were demonstrated in rat liver by Western blots that utilized a monoclonal anti-ER antibody (Alexander, et al., 1990). This method is more specific because it is based on epitope recognition by the antibody and therefore would be less likely to crossreact with other estrogen binders. It is more sensitive and therefore requires fewer mice, is less time consuming than the biochemical assays, and has the additional advantage of not involving the use of radioactive compounds. In addition, previous electrophoretic characterization of murine ER has been conducted exclusively in peripheral target tissues. Therefore, the Western blot experiments would provide data characterizing neural ER in the mouse, in addition to analyzing ER-NM interactions in the mouse brain, thus filling a gap in the literature.

Immunodetection Studies

Methods

Based on the preceding studies, females received injections of EB 12 hours prior to sacrifice. In addition, some females received oil vehicle injections to demonstrate that ER association with NM was dependent upon estrogenic stimulation. NM was prepared from 15 mice per experiment and aliquots containing an average of 6 μg DNA were loaded on the gel.

Gel electrophoresis and Western blots. Nuclear matrix samples were sonicated in Tris-SDS and dissolved in SDS-PAGE sample buffer by boiling for 5-10 min. Final concentrations in the sample buffer were 62.5 mM Tris-HCl pH 6.8, 5% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue. Molecular weight marker proteins (ECL markers, Amersham) used were: phosphorylase b (mol. wt. 97000), bovine serum albumin (mol. wt. 68000), ovalbumin (mol. wt. 46000), carbonic anhydrase (mol. wt. 31000), trypsin inhibitor (mol. wt. 20100), and lysozyme (mol. wt. 14400). Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% Tris-HCl Ready gels (Biorad) using the Biorad

Miniprotean II system and were transferred to nitrocellulose with the Biorad Transblot system following the manufacturer's protocols. The transfer was carried out overnight at 30 volts (90 mAmp maximum) in buffer containing 0.192 M Tris, 0.025 M glycine, 20% methanol, and 0.01% SDS, pH 8.3. Subsequent procedures were carried out at room temperature.

To confirm efficiency of the transfer, nitrocellulose was stained with Ponceau S (Sigma) and gels were stained with Coomassie blue. The lanes containing the molecular weight markers were cut from the blot and stored in Tris buffered saline containing 0.1% Tween 20 (TTBS). The blots were incubated in 5% blocking solution (5% nonfat dry milk in TTBS) for 1 hr at room temperature, rinsed twice with TTBS, and incubated in 1% blocking solution containing 22 ng/ml ER715 (a gift from National Hormone and Pituitary Program, National Institutes of Health) in the presence or absence of 1 μg/ml of the peptide used to raise the antiserum. Antisera ER715 was produced utilizing a synthetic peptide corresponding to amino acids 270-284 of the rat ER (Furlow, Ahrens, Mueller, and Gorski, 1990). This sequence is localized in the hinge region of the rat ER, is 100% homologous to mouse ER hinge region, and no similar sequences were present in other steroid hormone receptors (Furlow, et al., 1990). Blots were

washed twice for 10 min. in TTBS and incubated in 1% blocking solution containing 1:2000 peroxidase-linked donkey antirabbit $F(ab')_2$ serum (Amersham). Blots were washed once for 20 min. and 4 times for 5 min. in TTBS. Immunoreactive bands were visualized on Hyperfilm (Amersham) using enhanced chemiluminesence (ECL; Amersham). Films were scanned using an LKB Ultroscan XL scanning laser densitometer.

Results and Discussion

A representative Western blot of NM of EB-treated female mice is depicted in Figure 12. It revealed an immunoreactive band with an approximate molecular weight (M_r) of 68 kDa (lanes 3 and 4), corresponding to the reported M_r of ER. Two additional immunoreactive bands of approximately 46 and 97 kDa (lanes 3 and 4) were also present. These three immunoreactive species (68, 46, and 97 kDa) represent 23%, 29% and 48% of the total receptor population, respectively. Immunoreactivity of all three bands was inhibited by the presence of 45-fold excess of the peptide antigen used to raise the antisera (lanes 1 and 2), thereby demonstrating specificity of the interaction. In Figure 13, a representative blot comparing immunoreactivity of NM of EB- and oil-treated female mice

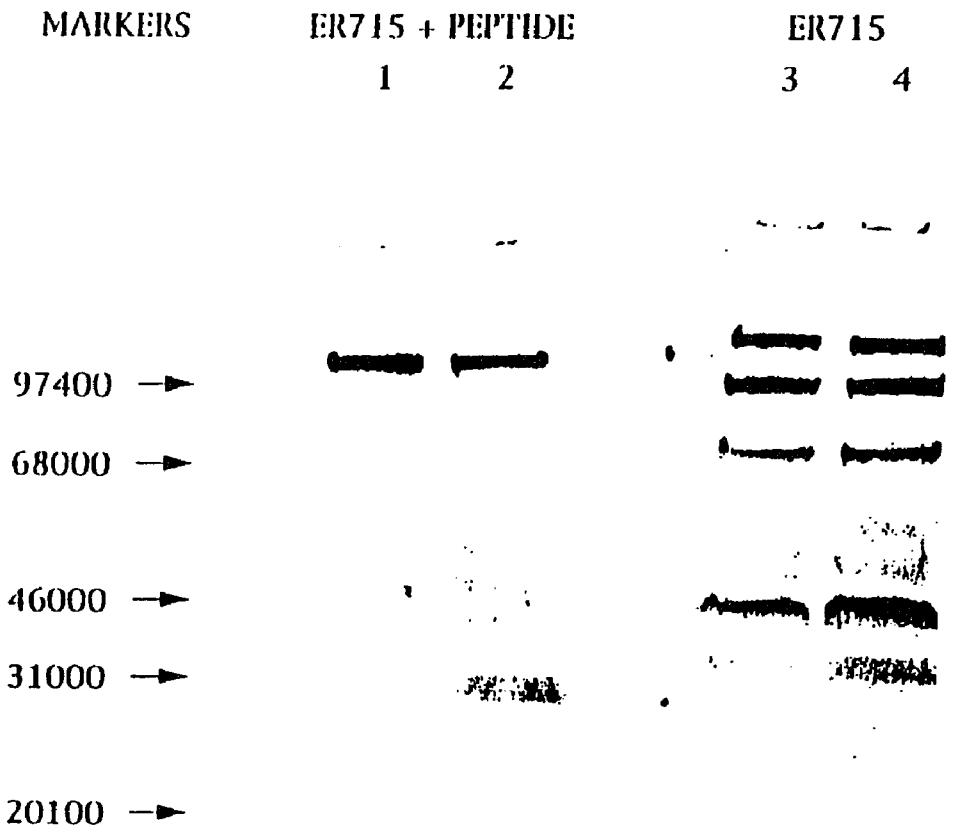


Figure 12. A representative Western blot of NM from EB-treated female mice. Each lane contained 6 μg of DNA (nuclear equivalent). The three immunoreactive bands of approximately 46, 68, and 97 kDa present in lanes 3 and 4 were suppressed in the presence of a 45-fold excess of the peptide antigen present in lanes 1 and 2.

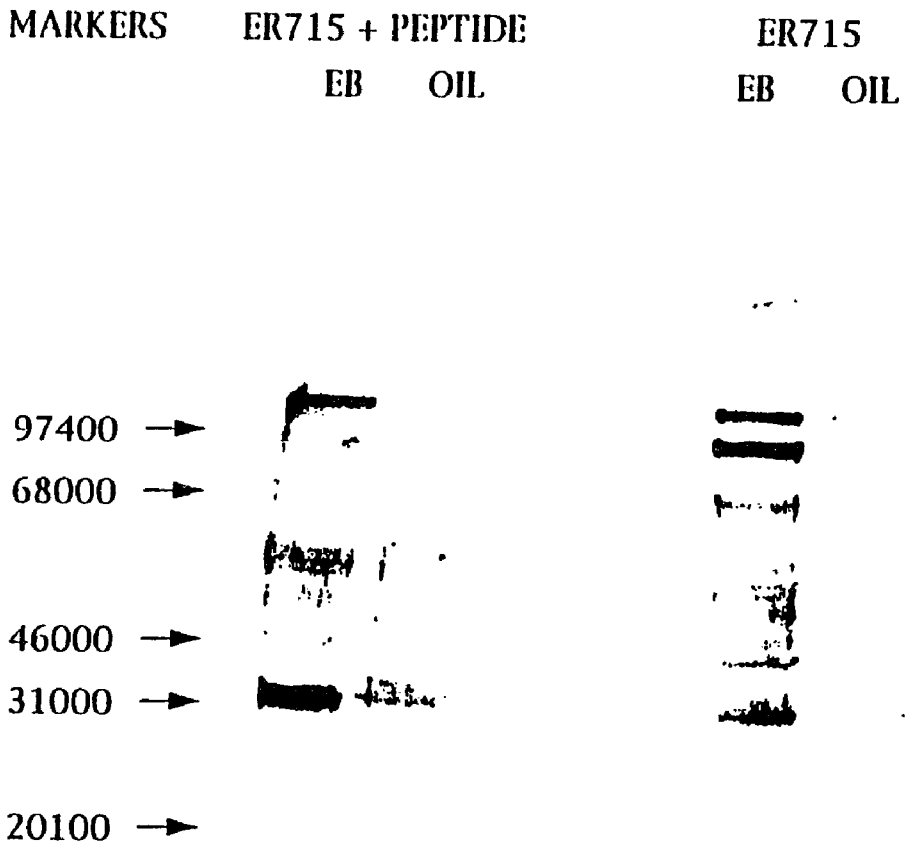


Figure 13. A representative Western blot comparing immunoreactivity of NM from EB- and oil-treated female mice. Each lane contained 6 μg of DNA (nuclear equivalent). The three immunoreactive bands of approximately 46, 68, and 97 kDa present in lanes 3 and 4 were suppressed in the presence of a 45-fold excess of the peptide antigen present in lanes 1 and 2.

demonstrated that the presence of the three immunoreactive bands was dependent upon exposure to EB *in vivo*. Densitometric scans of the blots of EB- and oil-treated females are depicted in Figure 14. The 46, 68, and 97 kDa bands correspond to the peaks at positions 152-154, 164-166, and 168-170 mm, respectively.

The only published report of NM-associated ER, from a study of the rat liver, yielded a single immunoreactive band of 67 kDa utilizing the monoclonal antibody H222 (Alexander et al., 1987). While an immunoreactive species in this range was detected in the present study, it comprised only 23% of the total receptor population. The 97 kDa species comprised 48% of the total population and this was consistent with existence of the 97 kDa species as the sole species detected in purified uterine and limbic nuclei depicted in Figure 15. These results differ from previous electrophoretic characterization of ER from several species and tissues which yielded major bands in the range of 67-68 kDa with numerous minor bands in the range of 30-54 kDa that are presumably proteolytic artifacts. However, a number of methodological differences exist between the past and present studies. For example, previous studies utilized estradiol (E_2)-affinity purified cytosolic preparations from adult animals (Lubahn, McCarty, and McCarty, 1985; Horigome, Golding, Quarmby, Lubahn, McCarty, and

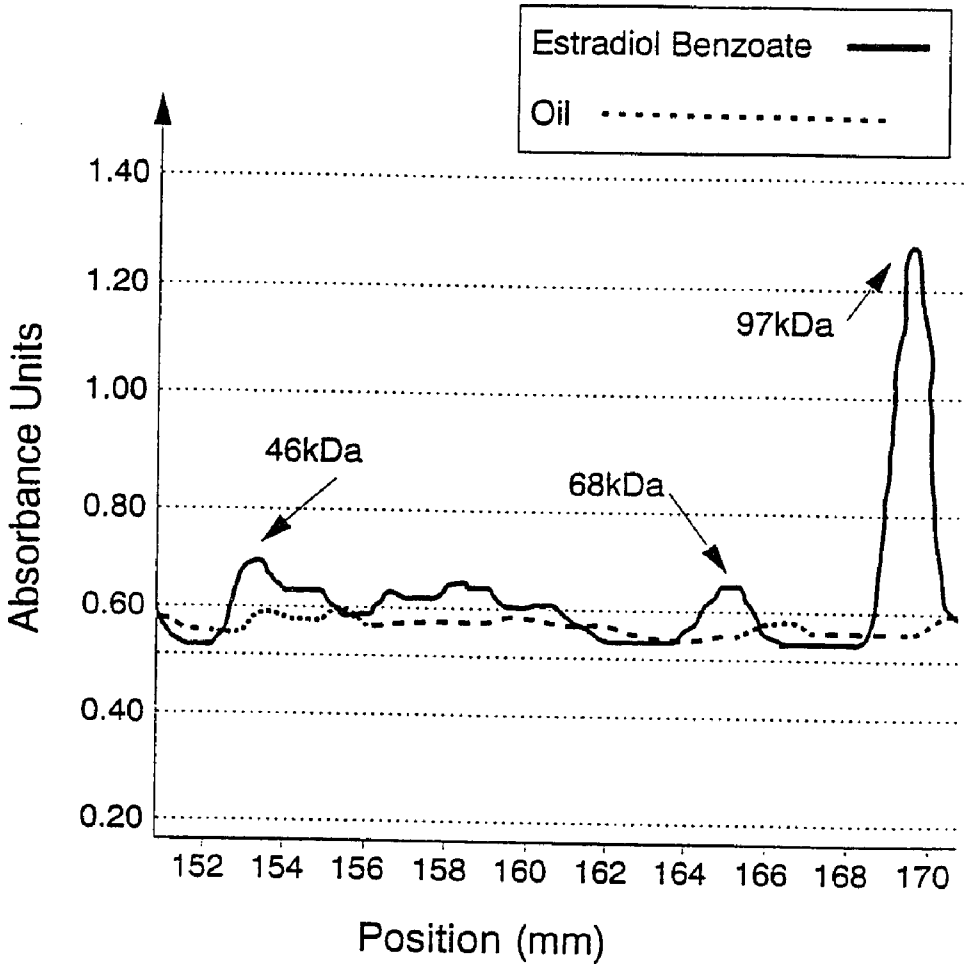


Figure 14. Densitometric scans of the blot in Figure 12 comparing NM from EB- and oil-treated female mice. The 46, 68, and 97 kDa bands correspond to the peaks at positions 152-154, 164-166, and 168-170, respectively.

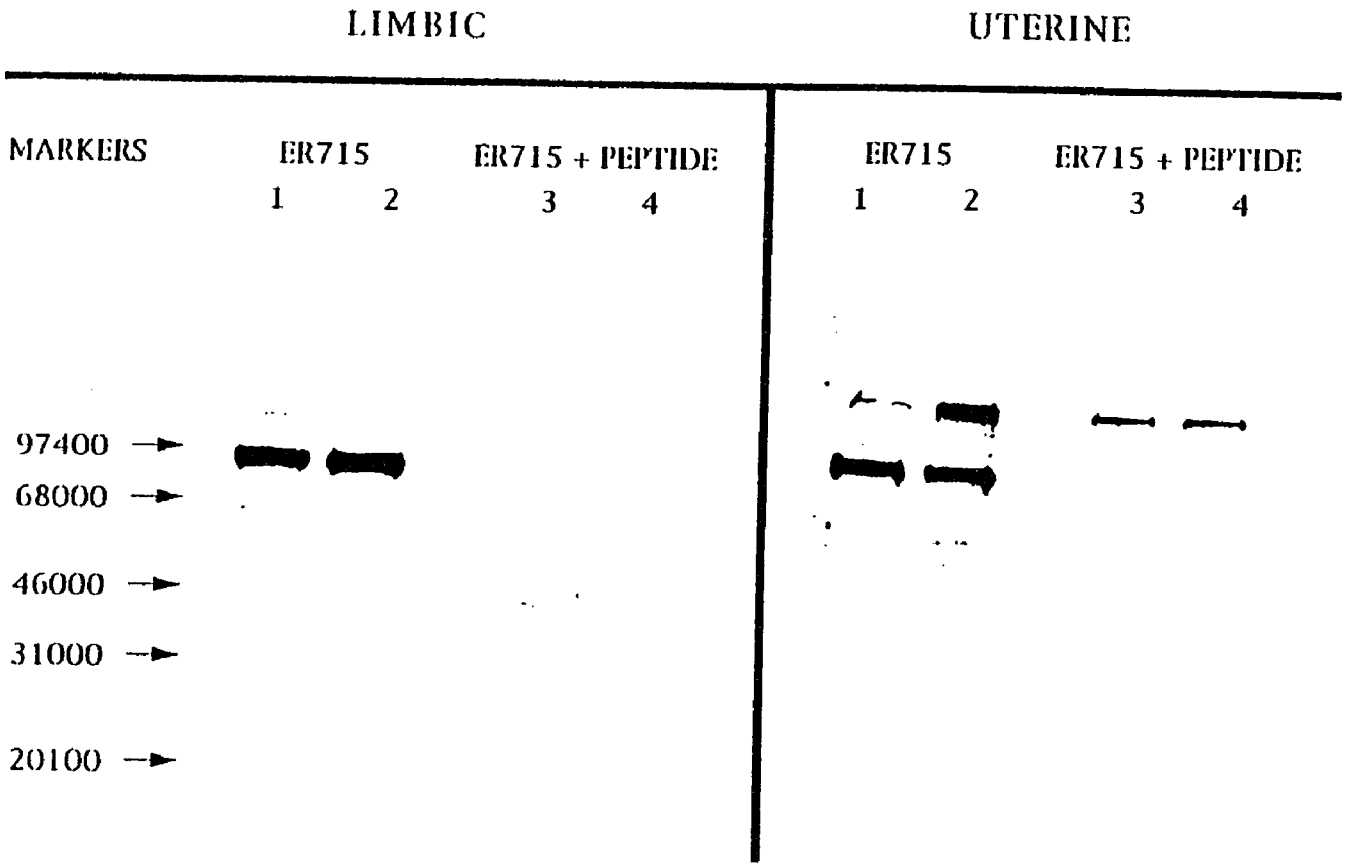


Figure 15. A representative Western blot comparing immunoreactivity of limbic and uterine nuclei from EB-treated female mice. Each lane contained 6 μg of DNA (nuclear equivalent). The 97 kDa immunoreactive band present in lanes 1 and 2 in both preparations were suppressed in the presence of a 45-fold excess of the peptide antigen present in lanes 3 and 4.

Korach, 1987). The extensive processing of ER during affinity purification may have resulted in proteolytic degradation of a high M_r ER species and/or this immunoreactive species may not interact with E_2 with sufficient affinity to withstand the purification process. Other studies have analyzed ER in fetal and neonatal reproductive tissues, and it is possible that this high M_r ER species may not be present in the tissues of immature rodents (Greco, Furlow, Duello, and Gorski, 1991). Further, previous electrophoretic characterization of ER utilized covalent affinity-labeling with tamoxifen aziridine (TAZ) and immunodetection with the monoclonal antibody H222, raised against a region of the steroid binding domain of the human ER (Lubahn, et al., 1985; Horigome, et al., 1987; Faye, et al., 1986; Greco, et al., 1991). The high M_r immunoreactive band detected in the present study may not interact with TAZ and H222. ER has not been previously characterized in neural tissue of the mouse, however, a 67 kDa species was reported in whole tissue extracts of the rat hypothalamus (Zhou, Shughrue, and Dorsa, 1995). The reasons for this discrepancy are unclear, and the possibility that the 97 kDa species identified in the present study is an artifact needs to be ruled out by further analysis.

Interestingly, there have been previous reports of ER with molecular weight larger than 67 kDa. For example, an 89 kDa ER was

identified in the calf uterus (Atrache, Raatajczak, Senafi, and Hahnel, 1985) and an 80 kDa immunoreactive ER species was recently identified in a subclone of MCF-7 cells (Pink, Jiang, Fritsch, and Jordan, 1995). Although solubilization of the NM with SDS-PAGE sample buffer should have resulted in the dissociation of any noncovalent interactions between ER and NM components, it is plausible that the 97 kDa form in the present study represents an ER complexed to a nuclear protein that resisted dissociation. Support for the existence of an ER-protein complex in this molecular weight range comes from a report of a 100 kDa immunoreactive complex isolated from MCF-7 cells following chemical crosslinking *in vivo* (Rossini and Camellini, 1994). Dissociation of the complex revealed the presence of ER and a protein with a molecular mass of approximately 50 kDa (Rossini and Camellini, 1994). The nature and function of the 50 kDa protein is unclear, however, a protein of similar molecular weight ($\approx$ 45 kDa) that promoted ER-estrogen response element interactions in yeast has also been described (Mukherjee and Chambon, 1990). The nature of the 97 kDa species detected in purified nuclei from both uterine and limbic tissues is unclear. Nuclear ER in these preparations must be further characterized, however, this has been a problem due to the fact that commercially available monoclonal antisera are derived from

mice, and are not suitable for use in murine tissues (because this necessitates the use of anti-mouse secondary antibodies which will produce significant amounts of nonspecific interaction in mouse tissues). Preliminary data using different rabbit anti-ER polyclonal antisera (antisera 986, raised against a 38 kDa peptide from the N-terminal half of ER containing a portion of the A domain, in addition to the B, C, and D domains of the human ER; a gift from H. Ahrens) has confirmed the presence of a 97 kDa band in whole tissue extracts of the female mouse brain (unpublished data). Research in this area is ongoing.

CHAPTER FOUR: SUMMARY AND CONCLUSIONS

The Nuclear Matrix as Acceptor Site

The involvement of the NM in DNA organization, replication, and transcription, coupled with the correlation between hormone treatment and the stimulation of RNA synthesis and DNA replication, led investigators to examine the role of the NM in SR function shortly after the identification of the NM in 1974 (Berezney and Coffey, 1974). At the time there was evidence that SR bound to nuclear "acceptor sites" in a saturable, high affinity, tissue-, and steroid-specific fashion. In addition, SR bound to these acceptor sites were resistant to solubilization with salt. The fact that the NM was a salt-resistant structure led some investigators to examine the SR binding capacity of the NM. This resulted in the demonstration of saturable, high affinity, tissue-, and steroid-specific binding sites in NM derived from a wide variety of peripheral target tissues in several species.

There are a wealth of data implicating the NM as the nuclear acceptor site, however, the nature of the acceptor site is still unclear. The acceptor site may consist of NM-associated DNA, protein, or a combination of the two. Recent data points to the importance of protein-protein interactions, however, to date all models are purely

speculative. Spelsberg and colleagues have identified an acceptor protein for avian PR, RBF-1, that is necessary to generate specific association of PR with genomic hen DNA (Schuchard, Rejman, et al., 1991; Schuchard, Subramanian, et al., 1991). The localization of RBF-1 binding motifs on DNA derived from hen oviduct NM suggests that the interaction of PR with NM may involve RBF-1 (Schuchard, Subramanian, et al., 1991). This interaction may serve to localize PR on the NM such that a high concentration of PR are available to interact with PREs that may or may not be localized on the NM *in vivo*. Recently, RBF-1 has been detected in varying concentrations in numerous avian tissues including heart, lung, brain, kidney and pancreas (Landers, Subramaniam, Gosse, Weinshilboum, Madden, and Spelsberg, 1994). The ubiquitous nature of RBF-1 suggests the possibility that RBF-1 activity is not restricted to PR-DNA interactions but may play a role in mediating SR and/or other nuclear transcription factor interaction with DNA.

The resistance of acceptor sites to DNase I digestion provides further support for the role of proteins in the composition of the acceptor site. For example, rat prostate NM prepared by extensive DNase digestion that removed 99.1% of the nuclear DNA contained 50% of the acceptors present in the nucleus compared to 68% when

NM was prepared following standard, less extensive DNase digestion (Barrack, 1987b). In addition, it was recently demonstrated that the DNA-binding domain of the human AR (hAR) was not necessary for AR-NM interaction in COS-1 cells, whereas, deletion of the C-terminal domain of hAR abolished AR-NM interactions in "depleted" matrices (van Steensel, Jenster, Damm, Brinkmann, and van Driel, 1995). Interestingly, the DNA-binding domain of the glucocorticoid receptor (GR) was necessary for GR-NM interactions in COS-1 cells, whereas the C-terminal domain seemed to be necessary for optimal GR-NM interaction, but was not absolutely required (van Steensel, Jenster, et al., 1995). Differences in the behavior of the AR and GR mutants indicate that there may be different modes of molecular interaction between SR and NM for each SR type (van Steensel, Jenster, et al., 1995).

Characterization of the molecular interactions between SR, NM, and candidate acceptor proteins will significantly enhance our understanding of the role of NM in steroid regulated gene expression. An important first step would be to determine whether or not SREs are associated with the NM. However, presence of SREs on the NM cannot be taken as evidence of their involvement in SR-NM interactions. This could be assessed directly through the use of

antisense probes that would block SR-SRE interaction, enabling the determination of the role of the SRE in the localization of SR on the NM. Next, mutational analyses of acceptor proteins such as RBF-1 could be conducted to determine their role in mediating SR-NM interactions. It has been suggested that there exists a conserved family of acceptor proteins for SR (Zhaung, et al., 1993). If this is the case, the cDNA for RBF-1 could potentially be used to isolate additional candidate acceptor proteins. In addition, crosslinking studies of NM-localized SR may lead to the identification of other candidate acceptor proteins.

Analysis of Steroid Receptor-Nuclear Matrix Interactions in Neural Tissue

The above discussion highlights the extent of our knowledge regarding SR-NM interactions in peripheral tissues and cell lines. Remarkably, research regarding SR-NM interaction in neural tissue has lagged considerably behind. Two decades following the isolation of NM, with the exception of recent reports of corticosteroid receptor interaction with hippocampal NM (van Steensel, van Haarst, de Kloet, and van Driel, 1991), no one has examined SR-NM interactions in

neural tissue. This is remarkable given the role of the gonadal steroids in the development and expression of sexual dimorphisms in gonadal steroid sensitivity and the presence of hormonally regulated NMPs and their postulated role in tissue-specific gene expression (Getzenberg, 1994). Utilizing the protocol developed in this study, estrogen-dependent ER-NM interactions have been demonstrated in the female mouse brain. This establishes a level of analysis for SR function in neural tissue that was previously unavailable.

NM prepared from brain regions known to regulate biobehavioral responsiveness to hormones can be assayed for sex differences in SR-binding capacity. These data could be used to determine whether sex differences in this measure correlate with sex differences in target tissue responsiveness to steroid hormones. In addition, these brain regions can be assayed following manipulation of the perinatal hormonal environment to determine if this affects the quantity or quality of SR-NM interactions. These analyses in neural tissue may be conducted via biochemical exchange assay if sufficient numbers of animals are used and if the appropriate precautions are taken to guard against interference by the type II estrogen binding site. In addition, the relationship between NM binding capacity and the protein constituents of the NM can then be addressed by two-

dimensional electrophoresis as previously done in peripheral targets and cell lines. These investigations of neural SR-NM binding may provide insights into the mechanisms involved in the sexual differentiation of neural tissue and the regional variability in steroid-regulated gene expression.

In addition to establishing the presence of estrogen-dependent ER-NM interactions in the mouse brain, the present study has raised the possibility of the existence of a high molecular weight ER of approximately 97 kDa. This immunoreactive species was first demonstrated in Western blots of NM preparations from EB-treated female mice. The existence of this species was then documented in Western blots of nuclear preparations from the uterus and limbic region of EB-treated female mice. Preliminary data utilizing different ER-specific polyclonal antisera has confirmed the presence of this high molecular weight ER in neural whole tissue extracts from EB-treated female mice. The validity of these findings will be further assessed following injection of female mice with different estrogens (e.g., diethylstilbestrol and estradiol), conducting Western blotting studies with various sources of antisera, and utilizing different nuclear solubilization procedures to ensure that the

presence of this 97 kDa immunoreactive species is not a procedural artifact.

ER were barely detectable on NM from ovariectomized oil-treated females, therefore it remains to be established whether this high molecular weight immunoreactive species exists in ovariectomized female mice. It will be interesting to ascertain whether this 97 kDa species is present in males, and whether there are sex differences in the proportion of the three immunoreactive species detected in neural NM. A thorough analysis of murine ER in neural and peripheral tissues is necessary to ascertain the validity of the 97 kDa immunoreactive species.

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VITA

Suzanne E. McKenna

Date and Place of Birth: October 29, 1966
New York, New York

Parents: Carol J. and Peter D. McKenna

Current Address: Office: Department of Biological Sciences
Iacocca Hall- 111 Research Drive
Lehigh University
Bethlehem, PA 18015

Home: 380 Minor St., Apt. 5
Emmaus, PA 18049

Education

Present — Lehigh University, Bethlehem, PA.
Ph.D. program in Behavioral Neuroscience.
Dissertation Title: Estrogen receptor-nuclear matrix interactions in the female mouse brain.

M.S. — Jan. 1992 Lehigh University, Bethlehem, PA
Thesis: An assessment of the agonist/antagonist effects of tamoxifen on the activation of sexual receptivity and the induction of hypothalamic progestin receptors in the female mouse.

B.A. — 1987-1989 Lehigh University, Bethlehem, PA

1984-1986 Emory University, Atlanta, GA

Honors

1995 — A.P.A. Dissertation Award

1994 — P.E.O. Scholar Award

1991-1993 — National Institute of Health Predoctoral Fellow in Biotechnology

1991 National Science Foundation Graduate Fellowship Competition- Honorable Mention

1987 Rhodes Scholar Candidate

Membership in Professional Organizations:

Phi Beta Kappa
Society for Neuroscience
Endocrine Society
American Psychological Society
American Association for the Advancement of Science

Professional Experience:

1995 Instructor, Department of Biological Sciences, Lehigh University

1994 Graduate Assistant, Central Animal Facility, Lehigh University

1990-1991 Graduate Assistant, Central Animal Facility, Lehigh University

1989-1990 Teaching Assistant, Department of Psychology, Lehigh University

Research Interests: Processes involved in mediating target tissue sensitivity to steroids; mechanism of action of antiestrogens; role of transcriptional activation functions in cell- and promoter-specific activity of agonists/antagonists.

Publications:

McKenna, S.E., Simon, N.G., and Cologer-Clifford, A. (1992). An assessment of the agonist/antagonist effects of tamoxifen in the female mouse brain. Hormones and Behavior 26, 536-544.

McKenna, S.E., and Simon, N.G. (1993). Genetic differences in progestin sensitivity in CD-1 and SW female mice. Physiology and Behavior 54, 167-170.

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McKenna, S.E., and Simon, N.G. (1995) Estrogen receptor interactions with the nuclear matrix of the female mouse brain.

Smoluk, S., Ferretti, M., McKenna, S.E., Lu, S., and Simon, N.G. (1995). Modulation of aggression by serotonin 1A and 1B agonists. Pharmacology, Biochemistry, and Behavior, submitted.

Ferretti, M., Smoluk, S., McKenna, S.E., Lu, S., and Simon, N.G. (1995). Serotonin 1A agonists facilitate copulatory behavior in male mice. Physiology and Behavior, submitted.

Lu, S., Nau, G., McKenna, S.E., Burton, J., and Simon, N.G. (1995). Androgenic regulation of androgen receptor immunoreactivity in male and female mouse brain.

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McKenna, S.E., and Simon, N.G. Strain differences in progestin sensitivity in CD-1 and SW female mice. Eastern Psychological Association, Boston, MA, 1992.

McKenna-Repsher, S.E., and Simon, N.G. Estrogen receptor interactions with neuronal nuclear matrix in the female mouse brain. The Endocrine Society, Abstr 1728, Anaheim, CA 1994.

McKenna-Repsher, S.E., and Simon, N.G. Estrogen receptor binding to hypothalamic-preoptic-septal nuclear matrix in the female mouse brain. Conference on Reproductive Behavior, Bethlehem, PA, 1994.

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