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DEVELOPMENT AND REGULATION OF ESTROGEN RECEPTORS IN THE NEONATAL RAT HIPPOCAMPUS

by

Joan A. O'Keefe

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May

1992

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To Kevin, Sean Patrick and my parents

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VITA

The author, Joan Ann O'Keefe, was born on September 19, 1959 in Chicago, Illinois. In the fall of 1977 she entered the University of Illinois in Urbana, Illinois and graduated *Summa Cum Laude* with a Bachelor of Science degree in Physical Therapy in June, 1981. She was employed as a physical therapist from 1981 through 1986 and specialized in treating infants and children with neurological and musculoskeletal dysfunction.

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Joan O'Keefe successfully competed for a two year postdoctoral fellowship award for women in science from the University of Kentucky in Lexington. In April, 1992 she will begin this fellowship at the Sanders-Brown Center on Aging of the University of Kentucky under the supervision of Dr. Mark P. Mattson.

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LIST OF ABBREVIATIONS

AchE	acetylcholinesterase
AFP	alpha fetoprotein
AR	androgen receptor
BDNF	brain derived neurotrophic factor
Ca^{2+}	calcium
CAH	congenital adrenal hyperplasia
CAT	choline acetyltransferase
cDNA	copy deoxyribonucleic acid
CNS	central nervous system
DES	diethylstilbestrol
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
E	embryonic day
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response element
fra-1	fos-related antigen
GABA	gamma aminobutyric acid
GAP-43	growth associated protein 43 kilodalton
HPOA	hypothamalus/preoptic area
HSP	heat shock protein
5-HT	5-hydroxytryptamine
IEG	immediate early gene

IGF	insulin-like growth factor
LBD	ligand binding domain
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LTP	long term potentiation
kDA	kilodalton
Kb	kilobase
MAP	microtubule associated protein
MBH	medial basal hypothalamus
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NMDA	N-methyl-D-aspartate
PC12	pheochromocytoma
POA	preoptic area
PND	postnatal day
PVN	paraventricular nucleus
RNA	ribonucleic acid
RNAse	ribonuclease
SCG	superior cervical ganglion
SDN	sexually dimorphic nucleus
VMH	ventromedial nucleus of the hypothalamus

CHAPTER I

INTRODUCTION

Males and females in most vertebrate species, including humans, display marked differences in their behavioral repertoire. Many of these gender differences are demonstrated in behavior patterns important for reproductive function and perpetuation of the species. In the rat, for example, the male-typical mating response involves assumption of a mounting position, while the female posture is one of lordosis. In song birds, best represented by the zebra finch and canary, the male sings a courtship song prior to mounting and copulation, while the female never sings. Parental care is also predominantly performed by one sex in several species (Arnold, 1980; Kelly, 1988).

In mammals, gender differences exist in many non-reproductive behaviors as well. These include juvenile play fighting, aggression and certain cognitive functions. Play fighting is a form of juvenile social behavior which is present in humans, primates, rodents and ungulates (Beatty, 1984). In these species, juvenile males initiate and engage in "rough and tumble play" more frequently than females (Erhardt and Meyer-Bahlburg, 1981; Meaney et al., 1983; Meaney, 1988). Males also display more intraspecies aggressive behavior than females (Beatty, 1984; Maccoby and

Jacklin, 1974). In the cognitive domain, male rats have been shown possess greater complex maze learning skills than females, which may reflect better spatial mapping ability (Dawson et al., 1975; Joseph et al., 1978; Stewart et al., 1975; van Haaren et al., 1990). Similarly, human studies have shown that men possess better visuospatial ability than women, while females perform better on verbal tasks (Kimura and Harshman, 1984; Hines, 1990).

Gender differences in many juvenile or adult behaviors arise as a consequence of differential gonadal hormone exposure during perinatal life, when testosterone levels are greater in males than females (Corbier et al., 1978; Weiscz and Ward, 1980). Thus, castration of the neonatal male and testosterone administration to neonatal female rats results in a reversal of many normal phenotypic behavioral responses seen in adulthood (Gorski, 1979; Joseph et al., 1978; Meaney, 1988). Likewise, studies examining individuals who were exposed prenatally to synthetic sex hormones, or those with endocrinological disturbances, suggest that the perinatal gonadal hormone milieu regulates the development of sex-specific behaviors in humans (Ehrhardt et al., 1981; Hines and Shipley, 1984). For example, human offspring with congenital adrenal hyperplasia (CAH) demonstrate overproduction of adrenal androgens. CAH females display more male-typical play behavior than do control subjects (Ehrhardt et al., 1968; Ehrhardt and Meyer-Bahlburg, 1981).

The intraneuronal conversion of perinatal testosterone to estrogen and subsequent binding to high affinity intracellular estrogen receptors (ER) in the brain appears to be a requisite event for the development of many sexually dimorphic behaviors and functions. This current dogma was established following several different lines of experimental evidence in the rodent. First, intrahypothalamic implants of either testosterone or estradiol are equally effective in inducing masculine sexual behavior in gonadectomized neonates (Christensen and Gorski, 1978). Second, both estrogen and testosterone induced masculinizing effects are abolished by synthetic antiestrogen treatment (McDonald and Doughty, 1973; Sodersten, 1978). Third, inhibitors of the aromatization of testosterone into estradiol block or attenuate the effects of endogenous or exogenous testosterone (McEwen et al., 1977). Thus, estrogen and ER activation is a primary mediator of sexual differentiation of the brain.

Brain regions thought to mediate reproductive functions and behaviors, as well as areas subserving nonreproductive functions, are sexually dimorphic in their morphological organization. Further studies demonstrated that gonadal steroid hormone exposure during development permanently alters the morphology, neurochemistry and connectivity of neural substrates (Arnold and Gorski, 1984; Toran-Allerand, 1984; MacLusky and Naftolin, 1981). These

discoveries led to the now widely accepted theory that functional sexual differentiation has an anatomical basis.

The hippocampal formation is a telencephalic brain region which exhibits sex-specific dimorphisms in its morphology and function (Loy, 1986). For example, several studies in the rodent have shown that certain regions and cell types in the hippocampus are larger in males than females (Pfaff, 1966; Diamond et al., 1982, 1983; Wimer and Wimer, 1985). In addition, behaviors in which the hippocampus plays a key role, such as skills requiring spatial navigation (O'Keefe and Nadel, 1978), are learned at a faster rate and performed better in males than in females (Barrett and Ray, 1970; Krasnoff and Weston, 1976; Herman and Siegel, 1978). These gender differences, along with evidence for the role of perinatal gonadal steroids in establishing these sexual dimorphisms (Pfaff, 1966; Dawson et al., 1975; Joseph et al., 1978; Stewart et al., 1978), suggested that the hippocampus is sensitive to the organizational actions of gonadal steroids. Therefore, we hypothesized that the hippocampus contains ER during a perinatal, as yet undefined, "critical period" when sexual differentiation is thought to take place.

The first series of experiments performed for this dissertation evaluated the postnatal development of ER in the male and female rat hippocampus. ER numbers were also evaluated in other cerebral cortical regions, as well as in

the hypothalamus, for purposes of comparison. Saturation and competition analysis of ^{3}H-estradiol binding was performed in the hippocampus to determine receptor affinity and specificity, respectively. In addition, the first study examined the ability of the neonatal hippocampal ER to transform to the functional DNA binding state.

The second study in this dissertation was designed to investigate the development of ER mRNA levels as an index of ER gene expression in the neonatal and adult hippocampus. Comparisons between the ontogenetic profiles of hippocampal ER protein levels (obtained from the first study) and ER mRNA levels, allowed us to test the hypothesis that differences in ER protein levels during development are secondary to alterations in ER gene expression.

The third and final study in this dissertation involved an examination of the mechanisms by which neural ER are regulated during development. Our major interest was to distinguish between the possible intrinsic and environmental effects on the pattern of ER phenotypic expression. This issue is critical to understand the coordinated sequence of events that form the substrate for hormonal induction of sexual differentiation of the central nervous system (CNS). We employed fetal brain grafts to examine the commitment of neural tissue to a specific timetable for ER ontogeny. This transplant paradigm allowed us to assess whether hippocampal ER development is preprogrammed or whether epigenetic

factors modify the timing of ER ontogeny.

CHAPTER II

REVIEW OF RELATED LITERATURE

Sexual Differentiation of the Central Nervous System

The development of gender differences in the neuroendocrine control of reproductive functions and in various sexual and non-reproductive behaviors is referred to as sexual differentiation of the central nervous system (CNS). In most mammalian species this phenomenon results from gender differences in gonadal steroid hormone secretion during a critical developmental period (MacLusky and Naftolin, 1981; McEwen, 1983; Toran-Allerand, 1984). In rodents, males are exposed to higher testosterone levels than females during certain time points in the perinatal period (Pang, et al., 1978; Rhoda, et al., 1984; Weisz and Ward, 1980). It is widely accepted that testosterone exerts an organizational influence on a "plastic" CNS during this time which permanently alters neural circuits and their function (McEwen, et al., 1982; Toran-Allerand, 1984). Male rats castrated at birth display feminine patterns of sexual behavior and gonadotrophin secretion as adults (Gorski, 1979). Likewise, testosterone administration to neonatal female rats results in behavioral and functional responses in adulthood that are similar to males. Suppression or

elimination of feminine characteristics such as the cyclical release of luteinizing hormone (LH) is known as "defemininization", while the development and enhancement of male characteristics is referred to as "masculinization" (Beach, 1981). Femininization of the brain and the development of female behaviors is generally thought to occur passively in the absence of hormonal induction. However, this popular theory has been questioned by several lines of evidence which will be presented later in this review.

Organizational versus activational effects of gonadal steroids

Gonadal steroid hormones influence mammalian reproductive function (and possibly nonreproductive function as well) in two fundamental ways depending on the maturation state of the animal. Thus, the actions of gonadal steroids on brain function and ultimately behavior have been classified as either organizational or activational. Organizational effects occur during a sensitive, perinatal period when the brain is relatively undifferentiated (Phoenix et al., 1959). Gonadal steroids function at this time by permanently altering the pattern of sexual behavior and gonadotropin secretion expressed later in the adult. In rodents, perinatal exposure to gender specific levels of gonadal steroids determines whether the copulatory behavior typical for males, mounting, or the female-typical behavior,

lordosis, will be exhibited in adulthood (Sodersten, 1978). Similarly, differential gonadal hormone exposure during the perinatal period determines whether the pattern of gonadotropin secretion will be cyclical with periodic LH surges leading to ovulation in the female, or relatively constant as seen in the male (Dyer, 1984). The permanent nature of organizational effects implies either structural changes in the brain (Arnold and Gorski, 1984) or long term cellular changes such as alterations in neuronal responsiveness to gonadal hormones (i.e. change in receptor number or affinity) or in hormone metabolism (Beach, 1971).

In adult life, the expression of these sexually dimorphic functions and behaviors is dependent on appropriate levels of circulating gonadal hormones. For example, gonadectomy eliminates or reduces the frequency of sexual behavior, and subsequent hormone administration reinstates these behaviors in response to appropriate sensory cues (reviewed in Blaustein and Olster, 1989). Thus, estrogens and androgens activate cellular and molecular events associated with neurotransmission in the adult. Activational effects are transient alterations in previously established neural circuits. Thus, gonadal steroid hormones, acting through their respective receptors, function perinatally to organize the brain into the male or female phenotype and act during adult life to activate the neural pathways responsible for adult sexually dimorphic

behaviors (Arnold and Breedlove, 1985).

Classifying the effects of gonadal steroids into two separate categories has been useful to provide a theoretical scheme for sex hormone action on the brain. However, there is ample evidence that various neural developmental or growth processes are similarly affected by gonadal steroids throughout life. For example, enhanced dendritic arborization and synapse formation are effects of gonadal steroids in both the developing and mature brain (Matsumoto and Arai, 1979; Meyer et al., 1978; Carrer and Aoki, 1982; Nishizuku and Arai, 1981a; Clough and Rodriguez-Sierra, 1983; Garcia-Segura et al., 1986; Chung et al., 1988; Frankfurt et al., 1990). Hence, the actions of these hormones during perinatal life compared to those found in the adult may not be fundamentally different. Instead, they may represent a continuum of effects on a system of decreasing plasticity (Arnold and Breedlove, 1985).

Critical period(s) during development

The critical period for sexual differentiation of the brain is that time during development when the CNS is sensitive to the organizational actions of gonadal steroids. This period is commonly defined in relation to points associated with the control of gonadotropin secretion and reproductive behaviors. In the rodent the onset of the critical period is thought to occur around embryonic day 18,

just following Leydig cell differentiation and at the beginning of testosterone secretion (MacLusky and Naftolin, 1981; Weisz and Ward, 1981). Others have defined the beginning of the critical period in relation to gonadal steroid hormone receptor development (Fox, et al., 1978; Vito and Fox, 1982), when the brain becomes sensitive and thereby responsive to estrogen and/or testosterone. This would place the beginning of the critical period at embryonic day 15 or 16 in the rat hypothalamus\preoptic area (Vito and Fox, 1982) and embryonic day 18 or 19 in the rodent cerebral cortex (Gerlach et al., 1983) when ER are first detectable in these regions. In general, the end of the critical period for masculinization and/or defemininization of reproductive function and behavior is around postnatal day 10 in the rat, as permanent gonadal steroid effects are not typically seen after this time (Lobl and Gorski, 1974; Barraclough, 1961; Arnold and Gorski, 1985). However, many sexually differentiated CNS regions and their functions are not maximally sensitive to gonadal hormones at identical time points (MacLusky and Naftolin, 1981; Rhees et al., 1990; Breedlove and Arnold, 1983). For example, the development of masculine sexual behavior is most sensitive to the effects of gonadal steroids prenatally, while mechanisms controlling cyclical gonadotropin secretion and female copulatory behavior (i.e. defeminizing actions of gonadal steroids) are highly

sensitive to organizational influences in the first several postnatal days but are relatively unaffected prenatally (McEwen, 1983; Arai and Gorski, 1968). Therefore, there is probably more than one critical period for the sexual differentiation of the brain, depending on the specific behavior, developmental process or brain region in question.

Gender differences in gonadal hormone secretion

It is believed that two periods during which testosterone secretion is at its peak in males accounts for sexual differentiation of brain areas mediating sexual, and possibly other, nonreproductive functions. A prenatal stage occurs on day 18 of gestation when serum testosterone levels in males reach the highest values seen throughout the perinatal period and sex differences are greatest (Weisz and Ward, 1980). The other stage occurs during the very early postnatal period. Serum testosterone levels in neonatal male rats exhibit a dramatic surge 2 hours following birth and then rapidly decrease to values comparable to that of the near term fetal male by 4 hours following parturition (Corbier et al., 1978). Hypothalamic testosterone levels in neonatal male rats parallels the temporal pattern observed in the serum (Rhoda et al., 1984). In contrast, during the first 24 hours following birth, testosterone levels are undetectable in female rat serum and hypothalamic tissue (Rhoda et al., 1984) although other investigators have

reported low but measurable levels at this time (Weisz and Ward, 1980). More importantly, plasma testosterone levels remain higher in males than in females from day 18 of gestation through at least the 5th postnatal day, although there may be considerable overlap in individual values between the two sexes (Weisz and Ward, 1980; Dohler and Wuttke, 1975).

The fate of testosterone

Testosterone has several fates once it passes through the plasma membrane. It may bind directly to androgen receptors which exhibit a specific regional distribution in the CNS or it may be enzymatically converted into 17 beta-estradiol. This locally produced estrogen is then free to interact with ER if contained within the cell. Additionally, testosterone may be reduced by the intracellular enzyme, 5 alpha-reductase, into dihydrotestosterone (DHT) which also binds with high affinity to the androgen receptor (AR). The AR does play a role in masculinization of the brain which will be discussed later in this review.

The role of aromatase

The intracellular aromatization of testosterone to 17 beta-estradiol and its subsequent binding to high affinity estrogen receptors (ER) located in specific CNS regions

appears to be paramount for the sexual differentiation of many neural structures and functions (McEwen et al., 1975, 1977; Lieberburg et al., 1980; MacLusky and Naftolin, 1981; Toran-Allerand et al., 1980a,b). Local estrogen biosynthesis is carried out by the aromatase complex, a microsomal P450 enzyme system. In adult mammals, the aromatase enzyme is concentrated in the hypothalamus, the preoptic area and the medial and cortical nuclei of the amygdala (Roselli et al., 1985, 1987). This distribution pattern corresponds well with androgen-sensitive brain sites. Defemininization of gonadotropin secretion and sexual behavior are induced by placing testosterone (or estrogen) implants into the developing hypothalamus (Christensen and Gorski, 1978). In addition, several morphological and biochemical responses to androgen have been observed in brain regions with high levels of aromatase activity (Gorski, et al., 1980; Luine et al., 1975; Dorner and Staudt, 1968; Nichizuka and Arai, 1981). Brain regions such as the cerebral cortex and hippocampus do not appear to have appreciable levels of endogenous estrogen biosynthesis in adult rodents (Roselli et al., 1985; Loy et al., 1988).

The distribution of aromatase in the developing brain is similar to that seen in adulthood (George and Ojeda, 1982; Tobet et al., 1985) but aromatase activity is higher in the preoptic area and hypothalamus between embryonic day 18 and 20 than at any other time. This temporal pattern

precisely coincides with the prenatal surge in male testosterone output. Several investigations have failed to demonstrate evidence for estrogen biosynthesis in developing cerebral cortical tissue (George and Ojeda, 1982; Tobet et al., 1985). More recently, however, significant levels of aromatase enzyme activity have been reported in the hippocampus and in the orbital, dorsolateral prefrontal and anterior cingulate cortices of the developing rhesus monkey, as well as in the postnatal day 5 rat hippocampus (MacLusky, et al., 1987; Clark et al., 1988) and in the human fetal hippocampus (Naftolin, et al., 1975). Lower but detectable levels were also observed in the parietal, somatosensory, motor and visual cortices of fetal rhesus monkeys (MacLusky et al., 1987; Clarke et al., 1988). Local estrogen biosynthesis from the aromatization of androgens has also been observed in 3 day old organotypic cultures of the newborn mouse hippocampus and anterior cingulate cortex (MacLusky et al., 1986, 1987). Thus, the presence of aromatase activity in the developing hippocampus and cerebral cortex of the rodent and monkey, albeit lower than that seen in brain sites controlling reproductive function, suggests that androgen-derived estrogen may play a role in the sexual differentiation of these higher cortical structures.

Hypothalamic estradiol levels were found to be significantly greater in males rats than females with a

transient increase in levels at 1 -2 hours following birth (Rhoda et al., 1984). This surge was absent in females, and abolished in males by gonadectomy performed at the time of birth, which suggested that the estrogen was derived from the aromatization of testicular testosterone. Endogenous estradiol was not detectable in the cerebral cortex at this time which does support previous studies demonstrating lack of cortical aromatase at this time (George and Ojeda, 1982; Tobet et al., 1985). However, it does not rule out the possibility that aromatase activity is present at different times in the perinatal period. If this were the case, the cerebral cortex may have a different critical period for sexual differentiation than the hypothalamus. Alternatively, it is possible that circulating estradiol, other than that derived from the aromatization of testosterone, might be involved in cortical maturation.

The role of the androgen receptor (AR) in sexual differentiation

As stated previously, testosterone or its metabolite, DHT, may bind to the AR which also has been implicated in the sexual differentiation of neural regions controlling several sexually dimorphic behaviors and functions. These include the spinal motoneurons innervating perineal muscles involved in rodent copulatory behavior (Breedlove et al., 1982) and brain sites mediating the hormone-dependent differentiation of rodent play behavior (Meaney, et al.,

1983; Meaney, 1988). Past studies in the above systems have demonstrated a primary effect of the AR in organizing the development of these sexually dimorphic brain regions and the behaviors in which they participate. Thus, depending on the species, behavioral and structural masculinization for certain sexually differentiated functions may have a pure androgen component.

Femininization of the brain

Femininization is a poorly understood phenomenon but is generally believed to represent the nervous system's intrinsic organizational pattern which emerges passively in the absence of hormonal induction (MacLusky and Naftolin, 1981). However, this theory has not obtained universal support (Shapiro, et al., 1976; Dohler, 1978; Dohler et al., 1984; Toran-Allerand, 1984) and certain studies suggest that low estradiol levels during postnatal life are necessary for the feminization of sexual behavior and for female neural organizational patterns to emerge. For example, ovariectomy performed during the postnatal or prepubertal period alters the development of the feminine pattern of sexual receptivity (Gerall et al., 1973; Hendricks and Duffy, 1974; Hendricks and Weltin, 1976). Similarly, postnatal exposure of female rats to the estrogen receptor antagonist, tamoxifen, defemininizes gonadotropin regulation, alters the feminine structure of the sexually dimorphic nucleus of the

preoptic area, and inhibits female sexual behavior without causing masculine sexual behaviors to develop (Hancke and Dohler, 1980, Dohler et al., 1984). Simultaneous, low-dose estrogen administration prevents the tamoxifen-induced effects (Dohler et al., 1984). These studies suggest that the low amounts of estrogen the female brain encounters may be necessary for the full expression of female gonadotropin secretion and sexual behavior. The fact that ovariectomy during prepubertal life inhibits female sexual receptivity is interesting and suggests that the critical period for this behavior may extend well beyond that reported for other reproductive functions.

The femininization of certain nonreproductive functions and female cortical maturation may also require low estrogen exposure. Males and females display sex differences in activity and exploration. Monitoring an animal's behavior in an open-field is frequently used to assess the above variables. Ovariectomy of neonatal female rats has been shown to reduce their open-field activity to the lower level characteristic of the adult male (Blizard and Denef, 1973). Similarly, low "submasculinizing" levels of estradiol in neonatally gonadectomized males or females leads to the development of the high levels of open field behavior characteristic of the adult female, whereas high "defeminizing" estrogen doses administered before postnatal day 10 reduce female open-field activity when measured in

adult life (Stewart and Cygan, 1980).

Gender differences in the symmetry of the rat cerebral cortex have also been reported; the right hemisphere is thicker than the left in males and the converse is seen in females (Diamond et al., 1981). The functional significance of these anatomical sex differences is unclear but they might relate to gender differences in cognitive function. Early postnatal, but not adult, ovariectomy was found to reverse the female pattern suggesting that cerebral cortical maturation may be under a "femininizing" influence of low concentration estradiol exposure.

The above studies, combined with *in vitro* experiments demonstrating developmental neurotrophic effects of estrogen (reviewed in a subsequent section), suggest that certain aspects of both male and female nervous system development are induced by estrogen. Therefore, some sexual dimorphisms may arise from varying degrees of estrogenic stimulation.

The role of alpha-fetoprotein

The plasma protein, alpha-fetoprotein (AFP), binds estrogens with a relatively high affinity in the rodent, and was originally thought to protect the developing brain (especially that of the female) from placental, maternal, ovarian, or exogenous estrogens during the critical period for sexual differentiation (McEwen et al., 1975; Vannier and Raynaud, 1975). AFP is synthesized transiently in large

amounts during the latter part of gestation and the early postnatal period (Vannier and Raynaud, 1975; Raynaud, 1975). Since AFP does not bind androgens, testosterone (which is present in higher concentrations in the male) is free to enter the brain and initiate estrogen or androgen receptor-mediated events.

More recent investigations have suggested a role for AFP in sexual differentiation of the rodent CNS which is not protective in nature. An intraneuronal pool of AFP exists in the cytoplasm of the developing mammal (Attardi and Ruoslahti, 1976; Plapinger and McEwen, 1973; Toran-Allerand, 1980b, 1982). It is present in both sexes at developmental stages ranging from the postmitotic neuroblast to more differentiated neurons. Since AFP mRNA has not been found in the rodent brain (Schachter and Toran-Allerand, 1982; Sell et al., 1985), the protein is probably derived from extracellular sources. Indeed, the ability of developing neurons to internalize exogenous or hepatocyte-synthesized AFP has been demonstrated by its uptake in primary cultures of fetal mouse brain (Uriel et al., 1981; Toran-Allerand, 1987) and by autoradiographic localization of radiolabelled-AFP in the embryonic fetal rat brain following its injection into pregnant dams (Uriel et al., 1983). The above findings have suggested that AFP might function as a carrier protein mediating the intraneuronal transport of estrogen and thus, regulating the intraneuronal concentration of estrogen

and/or serving as an intracellular reservoir for estradiol (Toran-Allerand, 1984; Toran-Allerand, 1987). This putative role for AFP would be especially pertinent for neurons that lack significant amounts of aromatase activity but which do contain ER. It would also support the theory that femininization may be an estrogen-dependent phenomenon, as is masculinization, and that the two processes differ only in the relative amount of estrogen exposure.

Neurotrophic Effects of Gonadal Steroids

Many of the effects of gonadal steroid hormones are similar to those mediated by classical growth factors (Gorski, 1985, Toran-Allerand, 1984). The vast majority of information regarding gonadal steroid neurotrophic effects on the developing nervous system pertains to differentiation of CNS regions that underlie sex differences in gonadotropin secretion and reproductive behavior. There is a relative paucity of investigations examining direct effects of gonadal hormones on the development of other brain structures despite the abundant behavioral evidence for sex differences in cerebral function and nonreproductive behaviors (Harris, 1978; Beatty, 1979, 1984; Kimura and Harshman, 1984; Hines, 1990). Therefore, this section will provide a general review of the known developmental and trophic effects of gonadal steroids on brain structure and function including specific effects on the hippocampus and

cerebral cortex when pertinent and available.

Anatomical Effects. Numerous examples of morphological sexual dimorphisms exist as a consequence of perinatal gonadal hormone exposure. For example, gender differences in the volume of specific brain regions or nuclear groups with concomitant alterations in neuronal number arise secondary to differential sex steroid exposure during development. These anatomical sites include the rat sexually dimorphic nucleus of the preoptic area (Gorski et al., 1978, 1980; Jacobson et al., 1981), rat spinal motoneurons innervating perineal muscles involved in copulatory behavior (Breedlove and Arnold, 1980, 1983; Jordan et al., 1982), the rat superior cervical and hypogastric ganglia (Dibner and Black, 1978; Suzuki, et al., 1982, 1983; Wright and Smolen, 1983a,b), nuclei involved in the zebra finch songbird system (Nottenbaum and Arnold, Gurney and Konishi, 1980; Gurney, 1981) and the rat locus ceruleus (Guillamon et al., 1988).

Alterations in neuronal density or number during development could be secondary to reductions in naturally occurring cell death or alterations in neuronal proliferation or neurogenesis. In the motor neurons of the developing rodent spinal cord, experimental evidence strongly suggests that androgens operate in this manner by preventing cell death (Hauser and Toran-Allerand, 1989;

Nordeen et al., 1985b). In one study (Hauser and Toran-Allerand, 1989), this was evaluated by ^{3}H-thymidine labeling of motoneurons on the final day of mitosis in utero, and then examining androgen effects on the previously marked cells in organotypic cultures of fetal spinal cord segments. Since the cells were permanently labeled and neurogenesis was complete, the observation of greater thymidine labeled cells in androgen-treated versus control cultures indicated enhanced neuronal survival.

Increases in individual neuron size including the cell body, nucleus and nucleolus have also been reported following perinatal exposure of the CNS to sex steroid hormones. These include neurons in rat sexually dimorphic lumbar spinal motor nuclei (Breedlove and Arnold, 1980,1983; Jones et al., 1982), the avian brain vocal control system (Gurney, 1981), several nuclei in the preoptic area and hypothalamus of the rat and primate (Dorner and Staudt, 1968, 1969b; Hellman, 1976; Bubenick and Brown, 1973) and rat hippocampal dentate gyrus pyramidal cells (Pfaff, 1966). In addition, estrogen and androgen have been shown to cause alterations in several synaptic parameters in developing regions of the CNS (Ratner and Adamo, 1971; Raisman and Field, 1973; Matsumoto and Arai, 1979, 1981; Nishizuku and Arai, 1981, 1982; Guldner, 1982; LeBlond et al., 1982). Dendritic length and branching, dendritic spine number and density, and overall dendritic field distribution are

particularly responsive to the ontogenetic effects of gonadal steroids (Greenough et al., 1977; Meyer et al., 1978; DeVoogd and Nottebohm, 1981a; Gurney, 1981; Ayoub et al., 1982; Munoz-Cueto et al., 1990). It has been speculated that the wide array of potential anatomical gender differences induced by gonadal steroids form the substrate for sexually differentiated functions (Arnold and Gorski, 1984; Toran-Allerand, 1984).

The anatomical loci which exhibit sexual dimorphisms in one or more neuronal parameters is far more extensive than the sites listed above. However, it is not yet clear whether perinatal estrogens or androgens establish these sex differences. In the human, for example, sex differences have been noted in the size and shape of the corpus callosum (LaCoste-Utamsing and Holloway, 1982; Allen, et al, 1991) and the midsagittal size of both the anterior commissure (Allen and Gorski, 1986) and the massa intermedia (Allen and Gorski, 1987). The massa intermedia connects the two hemispheres at the thalamus and is more often present in males than in females (Rabl, 1958). Volumetric human gender differences also exist in certain nuclear groups in the preoptic-anterior hypothalamic area (Allen et al., 1989; LeVay, 1991) and in the suprachiasmatic nucleus (Swaab and Fliers, 1985). These anatomical sexual dimorphisms have been theoretically linked to sexually dimorphic functions, including the establishment of sexual orientation and

identity (LeVay, 1991; Gladue et al., 1984) and gender differences in neuropsychological and cognitive function (Hines, 1982, 1990; Hines and Shipley, 1983). The recent report of a significant size difference in an anterior hypothalamic nucleus between heterosexual and homosexual men, with no difference between presumed heterosexual women and homosexual men supports a biological basis for sexual orientation (LeVay, 1991). Not surprisingly, the hypothetical link between a biological or hormonal basis for homosexuality and sex differences in cognition is quite controversial and invokes the nature versus nurture debate.

In vitro models. *In vitro* model systems have provided additional information regarding the morphological and biochemical effects of gonadal steroids. Exposure of embryonic day 17 or newborn mouse hypothalamic or cerebral cortical explants to estradiol (or testosterone) promotes dose-dependent growth and arborization of neurites from explant regions specifically shown by autoradiography to contain ER (Toran-Allerand, 1980a, 1984; Toran-Allerand et al., 1980). Similarly, estradiol enhances neurite growth from tyrosine hydroxylase-containing embryonic mesencephalic neurons in culture (Reisert et al., 1987). Interestingly, estrogen treated preoptic area (POA) cultured neurons demonstrate an increase in the number of first-order dendritic branches (Toran-Allerand, et al., 1983), a primary

differentiation pattern which is thought to be intrinsically determined. This supports a genomic role for estrogen in CNS development. In summary, then, differences in gonadal steroid exposure may result in variations in the growth of target axons and dendrites and in their synaptic organization and stability. This could affect neuronal survival and ultimately result in sexually dimorphic neural circuitry.

The molecular effects of gonadal steroid hormones. The specific estrogen-orchestrated cellular and molecular events that stimulate neuronal growth and differentiation are just beginning to be elucidated. Estrogen enhanced neuronal growth may be mediated, in part, by its induction of several proteins intimately linked to key events in process formation and differentiation. For example, estrogen has been shown to promote an increase in the expression of tau, a microtubule-associated or stabilizing protein, in cultures of embryonic medial basal hypothalamic neurons (Ferreira and Caceras, 1991). This response proceeded and accompanied an increase in stable microtubules and neurite elongation. In the female adult rat ventromedial nucleus of the hypothalamus (VMH), estrogen positively regulates the expression of the growth-associated protein 43 kDa (GAP-43) gene, which encodes a phosphoprotein intimately involved in neurite outgrowth and synaptogenesis (Lustig et al., 1991). There was no effect of estrogen on the levels of GAP-43 mRNA

in the posterior hypothalamus or the frontal cortex, regions which have low ER numbers in the adult. In addition, male rats displayed greater GAP-43 mRNA levels than females in both the VMH and the cortex but this message was not found to be regulated by gonadectomy in males. Thus, estrogen may alter neurite outgrowth and synaptic plasticity through regulation of many neural development associated proteins, a group to which GAP-43 belongs. Sex differences in synaptic density or organization observed in several brain regions might reflect gender variations in the expression of transcripts such as GAP-43. However, it is not yet known if GAP-43 or other molecules intimately involved in process formation and synaptogenesis are developmentally regulated by estrogen during the establishment of sex-dependent differences in neural connectivity.

It is unclear whether estrogen's neurotrophic effects are direct or secondary to regulation of or interaction with intermediary growth factors. Estrogen has been shown to synergize with insulin and with insulin-related peptides in the enhancement of neurite outgrowth from preoptic area, hypothalamic, and cerebral cortical explant cultures (Toran-Allerand, et al., 1988; Toran-Allerand, 1989). Similarly, the estrogen-enhanced axonal growth observed in cultured embryonic hippocampal cells was also found to be dependent on interaction with unidentified factor(s) present in fetal calf serum; prior extraction of growth factors and hormones

from serum with charcoal-dextran abolished estrogen's neuritogenic effect (Blanco et al., 1990). Intraventricular administration of nerve growth factor (NGF) antiserum to estrogen treated neonatal female rats prevented estrogen's known defeminizing effect on adult lordosis behavior (Hasegawa et al., 1991). This finding suggests that NGF may mediate some of the actions of estrogen during sexual differentiation of the hypothalamus which controls this behavior.

The potential for direct interactions between estrogen, NGF and other "classic" neurotrophic factors during neuronal development does exist. A recent *in situ* hybridization study demonstrated the co-expression of the mRNAs and protein for the ER and NGF receptor (NGFR), as well as NGF and brain derived neurotrophic factor (BDNF) protein in several regions of the developing rodent brain including neurons in the hippocampus, cortical plate, septum, nucleus of the diagonal band, striatum and nucleus basalis (Miranda et al, 1991). In addition, estrogen has been shown to positively regulate NGFR mRNA in dorsal root ganglion neurons of adult rats (Sohrabji et al., 1991). Estrogen may thus regulate neuronal responsiveness to NGF, thereby affecting neuronal survival, growth and differentiation. Estrogen and NGF might also display synergistic activity as evidenced by an increase in the number of cells expressing ER mRNA in explant cultures of the newborn mouse septum

treated concurrently with estrogen and NGF (Toran-Alleran et al., 1991). It is not yet known whether this effect represented enhanced neuronal survival or increased expression of the ER gene.

Localization of CNS Estrogen Receptors

The ER has been localized in both the developing (Attardi and Ohno, 1976; Brown, et al., 1989; Gerlach, et al., 1983; Keefer and Holderegger, 1985; MacLusky, et al., 1979; Sheridan, 1979; Stumpf and Sar, 1978; Vito and Fox, 1982) and adult (Pfaff and Keiner, 1973; Rainbow, et al., 1982a, 1982b; Stumpf and Sar, 1978) rodent CNS. In these studies, the predominant estrogen-concentrating neurons were found in the preoptic area, medial basal hypothalamus, and corticomedial amygdala. This anatomical distribution coincides with the regions known to mediate many gonadal steroid dependent reproductive behaviors or functions (Davis, et al., 1982; MacLusky and Naftolin, 1981).

Interestingly, large numbers of ER have been located in the developing rodent cerebral cortex (excluding the hippocampus) of both sexes during the early postnatal period (Friedman, et al., 1983; Gerlach, et al., 1983; MacLusky, et al., 1979; Shughrue et al., 1990; Sibug et al, 1991). Investigations utilizing *in vitro* binding assays found peak receptor numbers between postnatal day 7 and 10 with a subsequent attenuation in ER levels following this time. A study of the density and topographical distribution of

estrogen target cells in the developing mouse cortex during the early postnatal period demonstrated extensive reorganization of the cortical ER system with brain maturation (Shughrue, et al., 1990). The results suggested that ER-containing neurons might be migrating from deep to superficial layers of primarily the cingulate and suprarhinal cortical regions during the first postnatal week. While the function of cortical ER is largely unknown, their presence in migrating cells during differentiation and establishment of connections suggests that estrogen may play a role in neuronal differentiation and connectivity in the cerebral cortex (Shughrue, etal., 1990).

One interpretation of the lack of significant cerebral cortical aromatase activity is that estrogen may not be involved in masculinization or defemininization per se but might instead mediate more generalized developmental processes which are due to circulating estrogen versus that derived from testosterone (Toran-Allerand, 78, 81, 84). The peak in cerebral cortical ER occurs during a phase of rapid neuronal growth and differentiation (Bass, et al. 1969). The elevated serum estrogen levels from maternal and placental sources observed in both sexes decrease rapidly in the first few days after birth but increase again in the second postnatal week (Dohler and Wuttke, 1975). This observation lends credence for possible estradiol interaction with cortical (and other) ER. Although

MacLusky, et al. (1979) have questioned whether cortical ER are functional, since they were unable to demonstrate their occupation by endogenous estrogen in female rats, ER-containing organotypic cultures of mouse cingulate/frontal cortex as well as dissociated cerebral cortical cell cultures respond to estradiol with enhanced radial neuritic growth (Toran-Allerand, 1984; Uchibori and Kawashima, 1985) and increased protein synthesis (Toran-Allerand, 1984; Toran-Allerand, et al., 1988). In addition, estrogen administration during the early postnatal period has been shown to enhance cortical myelination (Curry and Heim, 1966). These findings suggest that the receptors are functional and may stimulate maturational processes in the cerebral cortex.

The ontogeny of estrogen receptors (ER) in the hippocampal formation has not been previously examined. However, a recent study reported a high concentration of estrogen target cells in the entorhinal cortex of the 2 day old mouse (Sibug et al., 1991). Low ER concentrations have been found in the dentate gyrus, pyramidal cell layer of Ammon's horn, and subiculum of the adult rat through autoradiographic analysis (Loy, et al., 1988; Pfaff and Keiner, 1973) and *in vitro* binding assays (Rainbow, et al., 1982). A substantial concentration of ER-containing cells has been reported throughout the rostrocaudal extent of the adult female guinea pig hippocampus, with greatest numbers

of ER immunoreactive cells in the amygdalo-hippocampal transition zone, as well as moderate numbers of ER in cells of the infragranular zone of the dentate gyrus and in the subiculum (Don Carlos et al ,91). There may be sex differences in, and effects of estrogen on, adult hippocampal and entorhinal cortical ER, since an immunocytochemical study failed to observe ER-immunoreactive cells in these regions in the adult male mouse, but labeled cells were found in the hippocampus and entorhinal cortex of diestrous, virgin and pregnant females (Koch and Ehret, 1989). In an extensive autoradiographic study of ER in the adult rat hippocampus, Loy et al (1988) found estrogen-concentrating cells in the CA1 pyramidal cell layer, layer IV of the entorhinal cortex, and interneurons of the hilar infragranular zone and of the ventral subicular molecular layer. Studies utilizing immunoenzymatic (Maggi, et al., 1989) and *in situ* hybridization (Pelletier, et al., 1988) methodologies suggests that relatively high levels of the ER protein and its mRNA are present in the hippocampus of adult rats. However, a recent *in situ* hybridization study examining the distribution of ER mRNA in the adult rat brain only found moderate levels of message expression in the hippocampus (Simerly et al., 1990). The discrepancies between the above studies are unclear but may represent variations in methodologies. In addition, differences between ER mRNA and protein levels in the adult hippocampus

suggest that posttranslational mechanisms might be operating to reduce ER protein numbers.

Since other studies have demonstrated that gonadal steroid receptor concentrations observed in neonates may be quantitatively different from those seen in adult animals (Friedman, et al., 1983; Gerlach, et al., 1983; Handa, et al., 1988; Keefer and Holderegger, 1985; MacLusky, et al., 1979; Shughrue, et al., 1990; Vito and Fox, 1982), with considerable differences observed between the developing and adult cerebral cortex, it was intriguing to examine the possibility that the hippocampus might exhibit age-dependent changes in ER levels.

Hippocampal Structure and Function: Sexual Dimorphisms and Effects of Gonadal Steroids

The hippocampus is a component of the limbic system and is thought to serve several important neural functions including storage of new information during learning (Swanson et al., 1982; Squire and Cohen, 1984). This cortical structure exhibits sex-specific differences in several morphological, neurochemical and electrophysiological attributes. In addition, several behaviors or functions that are believed to be mediated by the hippocampus are sexually dimorphic. The sections which follow include an overview of these gender differences and the known gonadal steroid effects on hippocampal function.

Morphology

Pfaff (1966) reported a greater hippocampal cross-sectional area as well as larger dentate pyramidal neuron size in male rats than in females. This dimorphism is influenced by neonatal gonadal steroids as neonatal castration abolished the sex difference. Morphometric analysis has shown that the right hippocampal formation is thicker in both developing and adult male rats than in females (Diamond, et al., 1982,1983). Greater dentate gyrus granule cell size, number and density, with an apparent reduction in naturally occurring granule cell death, has been observed in male mice compared to females (Wimer & Wimer, 1985;Wimer et al., 1988). The above mentioned sexual dimorphisms parallel many previously reported gonadal hormone induced anatomical sex differences in CNS sites governing reproductive function. Furthermore, evidence for neonatal gonadal hormone regulation of morphological characteristics in certain neuronal subsets (Pfaff, 1966) suggests that the hippocampus of the postnatal rat is sensitive to the organizational influence of these hormones.

Juraska (1990) has reported that male rats of weaning age (postnatal day 25) display less granule cell dendritic branches than females. This finding might suggest that there are sex differences in hippocampal estrogen (or androgen) sensitivity, possibly reflected in receptor numbers. Alternatively, a relative variation in the amount

of perinatal hormone exposure between the sexes may be related to dendritic growth or pruning. The gender difference in dendritic density was found to be transient as it was not observed in adults. The reason for this is unclear; perhaps earlier hormone exposure established the dimorphism which was then modified by other epigenetic influences.

Biochemical Parameters

A number of neurochemical sexual dimorphisms exist in the adult rat hippocampus indicating that hippocampal neurotransmission or its sensitivity to other neuromodulators is intrinsically different between males and females. These biochemical parameters are listed in Table I [p. 36]. The possible role of neonatal gonadal steroids in establishing these neurochemical dimorphisms has not been investigated. However, in other neural systems, sexual dimorphic neurochemistry has been linked to neonatal estrogen or androgen exposure (Simerly, 1989) which may relate to functional gender differences in physiological responses and behavioral capacities (Simerly et al., 1989; Simerly, 1990). Thus, hippocampal physiology and the cognitive processes in which the hippocampus plays a crucial role may be different between males and females. Experimental evidence for this hypothesis will be provided later in this section.

TABLE I

Biochemical sexual dimorphism	*Reference*
Glucocorticoid R number (female > male)	Turner and Weaver, '85
Benzodiazepine R binding (male > female)	Shephard et al., '82
Glucocorticoid regulation of VIP-stimulated cAMP production (In females dexamethasone elevates, in males it suppresses the response)	Harrelson and McEwen, '87
Norepinephrine content (male > female)	Robinson et al., '85
5-HT synthesis (females > males)	Haleem et al., '90
alpha-adrenergic R numbers (males > females)	Orensanz et al., '82

Abbreviations:
R, receptor
VIP, vasoactive intestinal peptide
cAMP, cyclic 5', 3' adenosine monophosphate
5-HT, 5-hydroxytryptamine

Gender differences have also been reported in the development of hippocampal neurotransmitter systems. For example, cholinergic enzyme activity in the rat septohippocampal system matures earlier in the female pathway than in the male (Loy and Sheldon, 1987). Specifically, the activities of both the biosynthetic enzyme, choline acetyltransferase (CAT) and the degrading enzyme, acetylcholinesterase, are higher in Ammon's horn and the subiculum during the first several postnatal days in females than in males. Since NGF regulates the development of CAT activity (Williams and Rylett, 1990) and estrogen may regulate NGF (Wright et al., 1988; Hasewaga et al., 1991),

it is intriguing to postulate that the neonatal gonadal hormone environment is partially responsible for the development of this neurochemical dimorphism.

In the hippocampus of the adult rat, estrogen influences several neurotransmitter and neuropeptide systems. These are listed in Table II [p. 38]. Therefore, estrogen appears to regulate several aspects of hippocampal neurotransmission and neurochemistry in adult life. Although these affects appear to be activational, it is feasible that perinatal gonadal hormone exposure influences the development of these estrogen-responsive neural circuits.

TABLE II

Estrogen (E) effects on hippocampal neurotransmitters, neuropeptides, their receptors (R) or biosynthetic enzymes in adult female rats in vivo

Neurotransmitter System	*E Treatment*	*Reference*
Increases benzodiazepine R	acute (12-24 hrs)	Perez et al., '88
Decreases LHRH R	chronic (2wks.)	Badr et al., '88
Increases mu-opiate R	2 days	Martini et al., '89
Increases GABA R	chronic (2 wks.)	Maggi and Perez, '84
Decrease GABA R	chronic (1 mo.)	Hamon et al., '79
Enhance 5-HT_{1A} mediated responses in CA1 pyramidal neurons	3-6 days	Beck et al., '89; Clarke and Goldfarb,'89 Clarke and Maayani etal., '90
Increases nicotinic Ach R	chronic (2mo.)	Lapchak et al., '90
Increases CAT activity	acute	Luine, '85

Abbreviations:
GABA, gamma-aminobutyric acid
5-HT, 5-hydroxytryptamine
Ach, acetylcholine
CAT, choline acetyltransferase

Neuronal Growth

Given the well characterized neurotrophic effects of gonadal steroids, it is significant that gender differences in hippocampal neuronal plasticity have been found in the rat. For example, females demonstrate greater dendritic arborization and spine density in dentate gyrus granule cells following environmental enrichment than males (Juraska

et al, 1985, 1989). In addition, the axonal reorganization that occurs following partial hippocampal deafferentation in adult animals is sexually dimorphic. Specifically, sympathetic axons sprout and innervate the hippocampus following septal lesions and this sprouting response is greater in magnitude in females than in males (Loy and Milner, 1980; Milner and Loy, 1980). This anatomical dimorphism may have important behavioral ramifications since the recovery of radial maze performance following septal lesions, which is correlated with neuronal reorganization due to sympathetic sprouting, is also sexually dimorphic (Harrel and Parsons, 1988). The sex difference in sympathetic sprouting following septal lesions is due to an organizational influence of gonadal hormones as neonatal castration of males or testosterone administration to females reverses the response, while adult gonadectomy is without effect (Milner and Loy, 82). Therefore, it appears that hippocampal plasticity may be permanently influenced by earlier, sex steroid hormone exposure. However, it is not yet known whether these represent pure ER or AR-mediated effects or whether they are due to an interaction between the two.

Gonadal steroids have also been shown to influence the reactive outgrowth of commissural-associational fibers to the dentate gyrus following entorhinal cortical ablations in a sexually dimorphic manner (Morse et al., 1986). Adult

gonadectomy reduces this sprouting response only in females, an effect which is reversed following estrogen replacement. Interestingly, gonadectomy combined with adrenalectomy significantly impairs this response in males, but not females. This dimorphism has been suggested to reflect the divergent pattern of corticosteroid-modulated sprouting between the sexes (Scheff et al., 1988a, 1988b). Thus, it appears that gonadal hormones may interact with glucocorticoids in mediating sexually dimorphic plastic responses in the hippocampus. It is feasible that perinatal gonadal steroids may sensitize the hippocampus to later modulation by glucocorticoids. The report of greater glucocorticoid receptor binding sites in the hippocampus of adult female rats than males (which is not affected by adult circulating gonadal steroids) supports this hypothesis (Turner and Weaver, 1985).

Gonadal hormones also affect hippocampal plasticity in the prepubescent and adult rodent. There is a pubertal increase in dentate gyrus granule cell and CA1 pyramidal neuron dendritic spine density in developing male mice; this response is prevented in CA1 but not in the dentate gyrus by castration performed on postnatal day 20 (Meyer et al, 78). Similarly, apical dendritic spine density in the CA1 pyramidal cell of female rats is positively regulated by estrogen (Gould et al, 90) and naturally fluctuates over the estrous cycle with peak spine density observed during

proestrus when estrogen levels are highest (Woolley et al, 90). This is the same morphological response that has been reported for the rat ventromedial nucleus of the hypothalamus, a classic estrogen target tissue involved in reproductive behavior (Frankfurt et al., 1990). Thus, it appears that the adult hippocampus is sensitive to estrogen action. Since increases in dendritic spine density are thought to mediate learning and information storage (Greenough, 1984), these functions may be modulated by sex steroid hormones in the hippocampus of adult animals.

Past studies have demonstrated direct neurotrophic effects of gonadal steroids on developing hippocampal neurons. For example, estrogen enhances neurite outgrowth in rat fetal hippocampal cells in vitro; this effect was selective for axons and may be dependent upon interactions with growth factors present in calf serum (Blanco et al, 90). In addition, estrogen differentially modulated the pattern of Na^+, $-K^+$-ATPase isoform expression in the same culture system. This result adds a new facet to estrogen's role in hippocampal development as alterations in isoform expression of this protein are associated with neuronal differentiation. In addition, gonadal hormones may directly affect neurogenesis in the dentate gyrus because testosterone injections to mice on the first day of life increase the amount of ^3H-thymidine labelled cells on PND-5 compared to controls (Seress, 1978). This effect may

explain the greater number and density of granule cells observed in male mice than in females (Wimer and Wimer, 1985). Evidence for estrogenic regulation of growth, differentiation and neurogenesis in hippocampal neurons is significant as it demonstrates that these neurons are responsive to the organizational influence of estrogen and may thus contain ER during development.

Neurophysiology /Epilepsy

On a functional level, there are sex differences and gonadal steroid modulated alterations in the frequency of septal input which drives the hippocampal theta rhythm (Drewett et al, 1977) The hippocampal theta rhythm is a synchronized, slow rhythmical neuronal activity which appears to be crucial for normal hippocampal function in many animal species (Stewart and Fox, 1990). Disruption of this rhythm produces memory impairments that are similar to those seen after direct lesioning of the hippocampus (Mitchell et al., 1982). Thus, the theta rhythm is associated with learning and mnemonic function. Reports demonstrating that the frequency of this rhythm is optimal for long term potentiation (LTP), the long-lasting synaptic enhancement following patterned input, supports this hypothesis (Larson et al., 1986; Greenstein et al., 1988). The theta rhythm is dependent on signals arising from the medial septum which may be the "pacemaker" of the theta

rhythm (Stewart and Fox, 1990). The shape of the theta-driving curve which relates septal stimulus frequency and the threshold current for driving the theta rhythm in the hippocampus is different in male rats than in females (Drewett et al., 1977). The presence of testosterone in the male was shown to be responsible for this sex difference as gonadectomy with testosterone replacement alters the female pattern to that of the male and long term castration changes the male to the female pattern. While this study may reflect an activational influence of testosterone, one cannot rule out the effect of estrogens or androgens in setting up this dimorphism. This study also has important ramifications for gender differences in learning and information storage given the putative role of the theta rhythm in mnemonic function.

Sex differences have also been observed in hippocampal pyramidal cell membrane excitability in adult rats (Teyler, 1980). Specifically, in the hippocampal slice preparation, tissues from males and females demonstrate opposite alterations in membrane responses following gonadal hormone administration in vitro. Perhaps these differences reflect an influence of the perinatal gonadal hormone milieu on hippocampal neuronal membrane properties as estrogens and androgens have profound, relatively permanent effects on the ultrastructure and intramembranous protein particle density of neuronal plasma membranes (Garcia-Segura et al., 1988,

1989 a,b). Moreover, the sexually dimorphic organization of rat hypothalamic neural membranes is due to the developmental actions of both estrogen and androgen.

The interaction between gonadal steroids and epilepsy is of important clinical significance. The hippocampus has a low threshold for seizure activity and is involved in epileptogenesis (Gloor et al., 1982). Excess release of the excitatory amino acid (EAA) neurotransmitter glutamate, and alterations in the number of its receptors, particularly the NMDA receptor (Sloviter 1983), has been implicated in the pathogenesis of epilepsy (Farooqui and Horrocks, 1991).

The observation of gender differences in seizure susceptibility and evidence for estrogenic modulation of epileptic activity may potentially be mediated by the ER in both the developing and adult hippocampus. Sex steroid hormones have been shown to alter the seizure threshold in animal models of epilepsy. Estradiol, in particular, has an excitatory influence on the CNS, manifested by lowering seizure thresholds (Woolley and Timiras, 1962a; Teresawa and Timiras, 1962b; Buterbaugh and Hudson, 1991). In addition, seizure frequency varies over the reproductive cycle both in women (Backstrom, 1976; Schachter, 1988) and in female rodents used in animal models of epilepsy (Woolley and Timiras, 1962b). Seizure exacerbation is seen during periods of high circulating estradiol. Thus, the physiological sensitivity of the hippocampus may vary with

alterations in gonadal hormone levels. Moreover, neonatal estradiol administration to rats has been shown to accelerate the development of an adult-like seizure pattern in the cerebral cortex (Heim, 1966). In humans, men exhibit a greater incidence of electroencephalogram (EEG) abnormalities in the right temporal lobe than women (Rey et al, 1949) and sex differences in the incidence of epilepsy have been reported in children (Maccoby, 1966). It is feasible that perinatal estrogen might contribute to the establishment of these sex differences.

The recent observation that estradiol suppresses Na-dependent Ca^{2+} efflux from rat brain synaptosomes (Horvat, 1991) provides a potential mechanism for estradiol's promotion of seizure susceptibility as aberrant rises in intracellular calcium are excitotoxic resulting in a cascade of neurodegenerative events (Siesjo et al., 1989). Estradiol reduces seizure thresholds (Woolley and Timiras, 1962a,b), alters calcium homeostasis (Horvat, 1991), and changes hippocampal membrane excitability (Wong and Moss, 1991) in a rapid fashion, suggesting that non classical ER-mediated mechanisms might underlie these effects. However, since estrogen can permanently alter membrane ultrastructure and protein density (Garcia-Segura et al., 1988, 1989a,b), one cannot rule out a genomic ER effect as a mechanism for sex differences in seizure susceptibility and hippocampal membrane excitability, which perhaps involves alterations in

ion channel proteins.

Behavioral Parameters

The hippocampus mediates several behaviors and functions which are sexually dimorphic. For example, the hippocampus is a substrate for certain learning and memory functions (Friedman and Goldman-Rakic, 1988; Mahut and Moss, 1984; Squire and Cohen, 1984; Swanson, et al., 1982; Zola-Morgan and Squire, 1986) including spatial mapping of the animal's environment (O'Keefe and Nadel, 1978; Sutherland, et al., 1982; Morris et al., 1982; Kesner et al., 1991). Studies performed in many species, including humans, have shown that males perform better than females on tasks requiring spatial skills and their acquisition (Beatty, 1979, 1984; Maccoby and Jacklin, 74; Corey, 1930; Dawson et al, 75; Joseph et al, 78; Grossi et al., 1979). In rats, sex differences for maze learning can be reversed by neonatal, but not by adult, gonadal hormone manipulations (Joseph et al, 78) suggesting that sex steroids are involved in establishing the circuitry for spatial navigation during development. Interestingly, CA1 pyramidal cells, a subset of which are thought to be the "place cells" involved in spatial skill acquisition (O'Keefe, 1979), have been shown to contain ER in the juvenile rat (Loy and McEwen, 1988). In addition, hippocampal stimulation facilitates memory function for avoidance task paradigms in male rats but has

no effect on females (Soumireu-Mourat et al., 1980) suggesting that the "wiring pattern" in the brain for learning this task is different between the sexes.

Humans also exhibit sex differences in both spatial abilities and certain learning and memory tasks (Harris, 1978; Grossi et al, 1979; Kimura and Harshman, 1984). Men perform better on tasks requiring visuospatial skills than women. Moreover, women with congenital adrenal hyperplasia (CAH), a disease causing hypersecretion of adrenal androgens, show heightened performance on spatial abilities tests than control subjects (Resnick and Berenbaum, 1982), suggesting that the perinatal gonadal steroid hormone environment may play a role in establishing gender differences in spatial learning in humans.

Model for Steroid Hormone Receptor Action

The cascade of cellular events initiated by gonadal steroids during the process of neural sexual differentiation is not well understood. The general model for gonadal steroid hormone action involves free passage of hormone through the plasma membrane, hormone binding to stereospecific nuclear receptors resulting in receptor transformation, and receptor binding to specific acceptor sites on DNA which results in altered gene transcription, protein synthesis and cellular function (Jensen et al., 1982; Blaustein and Olster, 1989; Carson-Jurica et al., 1990). The unoccupied estrogen receptor exists as a soluble

form which has a low affinity for DNA and is thus loosely bound to nuclear chromatin. Following ligand binding it undergoes transformation to an active form which tightly adheres to DNA or chromatin (Jensen, et al., 1982; Grody et al., 1982). The unoccupied form has been termed the "cytosolic" form because it is found in the cytosolic fraction following cell fractionation procedures. Following addition of hormone to cells or to hormone-deficient animals, the now occupied receptor is found in the nuclear fraction subsequent to tissue homogenization and ultracentrifugation and must be extracted from DNA with elevated salt buffers (Puca, et al, 1970). It follows then, that transformation of the receptor from the cytosolic to the nuclear form is dependent upon circulating hormone levels. Following gonadectomy most of the receptor is in the cytosolic form, while during periods of elevated estrogen (or testosterone if the aromatase enzyme is present and functioning) a portion of the receptor is in the nuclear form (Anderson et al., 1973; Lieberburg et al., 1980; Roy and McEwen, 1977). The occupied or nuclear form acts as a ligand-activated transcription factor and is consequently required for the genomic effects of estrogen (Evans, 1988). The sensitivity of cells to estrogen action is reflected in the number of cytosolic receptors present (Blaustein and Olster, 1989).

Biochemistry, Genomic Organization, and Molecular Biology of the Estrogen Receptor

The estrogen receptor (ER) belongs to a superfamily of ligand-modulated nuclear transcription factors which directly link gene transcriptional responses to extracellular signals. Included in this group are the other steroid hormone receptors, the receptors for thyroid hormone, retinoids and vitamin D, the retroviral oncogene product, v-erb A, as well as additional proteins termed orphan receptors with significant sequence homology for which ligands are not yet known (Evans, 1988; Carson-Jurica et al., 1990). These receptors acquire increased affinity for specific sites on chromatin upon hormone binding which results in enhanced transcriptional stimulation or inhibition of hormonally regulated genes (Beato, 1989; Yamamoto, 1985). The DNA elements to which the activated ER binds are termed estrogen response elements (ERE). These elements act as enhancers by exerting their actions in an orientation independent manner when placed upstream or downstream from promoter elements (Kumar, et al., 1987).

The ER is a soluble, high molecular weight (approximately 65 kDa) intracellular protein that binds 17-beta estradiol with high affinity (Kd $< 10^{-10}$ M) and selectivity over other steroid hormones or estrogens (Jensen et al., 1982). The ER (and other steroid hormone receptors) exists as a hetero-oligomer in the absence of hormone and

sediments in sucrose gradients at a sedimentation coefficient of 8S. In the presence of estrogen, the receptor dissociates to give a smaller hormone-binding unit with a lower sedimentation coefficient (4S) than the unoccupied receptor. The receptor interacts with nuclei, chromatin, and DNA in its occupied state. Therefore, a high salt concentration is needed to elute the receptor from DNA secondary to its heightened affinity (Toft and Gorski, 1966; Jensen and DeSombre, 1973; Puca, et al., 1970). The addition of estrogen to cells or to estrogen-deficient animals results in rapid transformation of the receptor from an inactive to an active state. This process is referred to as receptor transformation or activation (Carson-Jurica et al., 1990)

It is now known that the untransformed ER exists as a multiprotein complex associated with heat shock protein 90 (hsp-90) which explains the higher sedimentation coefficient (8S) in the absence of hormone. Heat shock proteins are ubiquitous, abundant and highly conserved molecules which are present in all species from bacteria to mammals. These proteins exhibit an increased rate of synthesis in response to stress, most commonly heat shock. It is thought that the 8S ER form is unable to bind DNA due to masking of the DNA binding domain by hsp-90 (Baulieu, 1987). Estradiol binding to its cognate receptor results in dissociation of hsp90 from the complex which leaves an activated, 4S form that has

increased affinity for DNA (Skafar and Notidas, 1985). [See figure 1, p. 52.] Hsp90 removal may uncover the DNA binding domain of the receptor and/or cause a conformational change in the receptor, either of which would relieve receptor inactivation (Catelli, et al, 1985; Groyer et al., 1987; Sanchez et al., 1987). The predominant theory is that steroid-induced dissociation of hsp 90 is accompanied by an irreversible conformation in the receptor which is both conducive to DNA binding and prohibitive to hsp reassociation (Dalman et al, 1989; Picard et al., 1987; Schwartz and Mizukami, 1991).

Transition metal oxyanions, such as molybdate, prolong the hsp 90-receptor association (Sabbah, et al., 1987). They have a high affinity for sulfur and can form bridge structures between sulfhydryl moieties (Kay and Mitchell, 1968), which may be how the proteins in the complex are stabilized. Therefore, endogenous metal ions are thought to play a role in stabilizing hsp90-receptor interfaces. Recent evidence suggests that hsp90 may have a role other than suppressing receptor function by stearic hindrance. Steroid receptors, produced under conditions in which they do not associate with hsp90, lack transcriptional enhancement activity, and the subsequent ability of hormone ligands to activate transcription functions is compromised (Picard et al., 1990). Hence, hsp90 may facilitate the subsequent response of the receptor to hormonal induction.

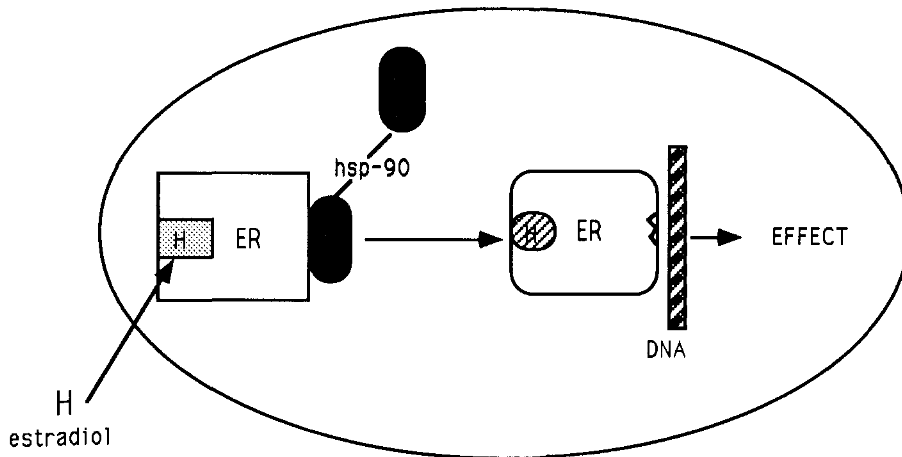


Figure 1

Schematic representation of estrogen receptor (ER) interaction with heat shock protein 90 (hsp90). In the absence of hormone (H), hsp-90 is associated with the ER; following ligand binding, hsp-90 dissociates, a conformational change occurs in the ER molecule, which is now transformed and able to interact with DNA regulatory elements.

The untransformed estrogen receptor, as well as other steroid hormone receptors, are also thought to complex with a variety of associated proteins such as the hsp-70 and hsp-56 (Sanchez, 1990) and other as yet undefined proteins (Smith et al., 1990). The function of the these additional proteins is not yet known. Interestingly, transformation of the glucocorticoid and progesterone receptor complexes to the DNA binding state results in dissociation of hsp90 but not hsp70 (Kost, et al., 1989; Sanchez et al., 1990), suggesting that activated steroid receptors may not be strictly monomeric complexes.

Biochemical characterization and cDNA cloning of the ER (and the other receptors in this family) has shown that the receptor protein is composed of six defined functional domains (A-F) [See figure 2, p. 54.] (Green, et al., 1986; Koike, et al., 1987; Kumar, et al., 1986; Kumar, et al., 1987). The amino-(N) terminal region A/B, or hypervariable region, is a poorly conserved one but it may be important for maximally stimulating transcription from certain estrogen responsive genes (Kumar et al., 1987; Lees et al, 1989). This region is also called the immunogenic domain as the epitopes of most antibodies that have been raised against steroid receptors are located here. This region may contribute to the diversity of specificity to the receptor.

THE ESTROGEN RECEPTOR DOMAIN STRUCTURE

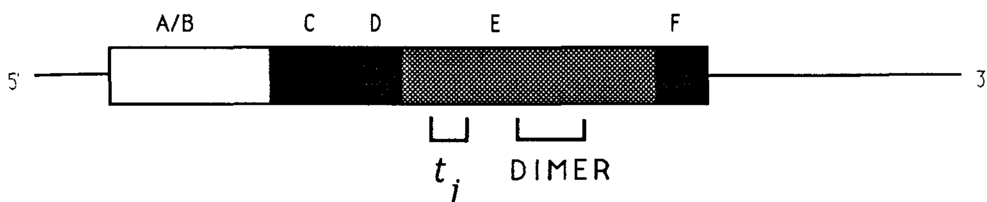


Figure 2

A/B: Hypervariable, amino terminal domain
C: DNA binding domain
D: Hydrophilic domain; contains nuclear localization signal sequence
E: Ligand or steroid binding domain; contains subdomains for hormone -relieved transcriptional inactivation (t i) and receptor dimerization.
F: Poorly conserved carboxy terminal domain

The single line refers to untranslated regions of the ER gene.

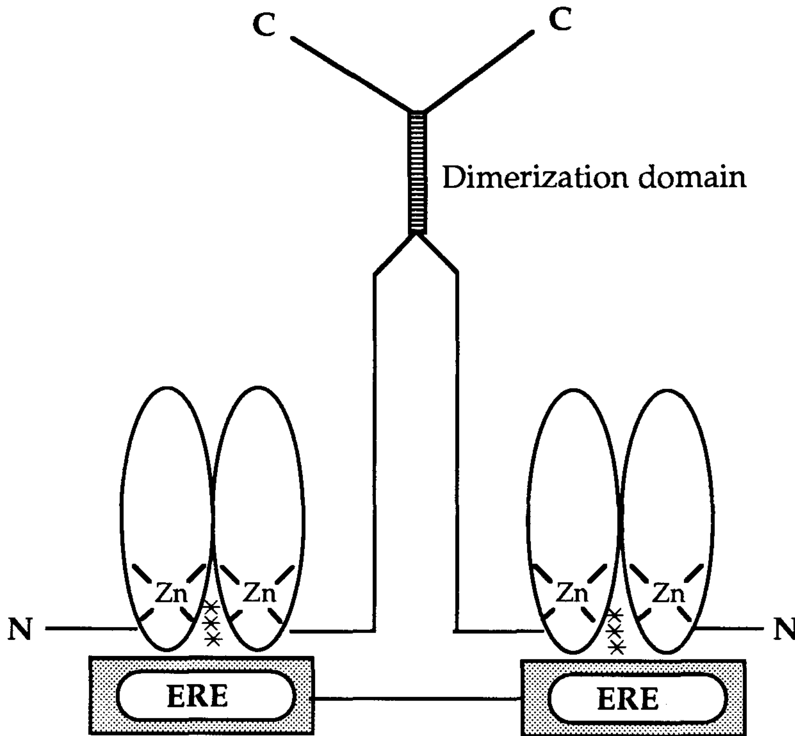


Figure 3

Depiction of a dimer of two estrogen receptors (ER) each bound to an estrogen response element (ERE) half-site. The DNA binding domain of each ER is represented by the zinc finger motifs (Zn). * refers to the three amino acids at the base of the first zinc finger which determine DNA binding specificity.

Region C is the DNA-binding domain and is important in determining target gene specificity (Green, et al., 1988). The general structure of this 68 amino acid region is highly conserved among members of the steroid hormone superfamily suggesting that the region performs important functions common to all of the receptors (Evans, 1988). Twenty invariant amino acids in this domain fold into two zinc-finger DNA binding motifs, each of which coordinates a zinc atom with four conserved cysteine residues (Freedman et al., 1988). [See figure 3, p.55.] The first finger is critical in determining target gene specificity due to its intimate involvement in response element recognition (Umesono and Evans, 1989).

Region D is a poorly conserved, hydrophilic domain which can be substantially altered by amino acid deletions or insertions without affecting ER function (Kumar et al., 1986, 1987). However, in the glucocorticoid receptor, this domain contains a 28 amino acid region termed nuclear localization 1 (NL1) which is the signal sequence required for steroid independent nuclear localization (Picard and Yamamoto, 1987). This region may be conserved in all steroid hormone receptors.

The steroid or ligand binding domain (LBD), region E, is believed to bind the ligand by forming a hydrophobic pocket (Green,et al., 1986). Region E also contains a subdomain for dimerization of the receptor (Fawell et al.,

1990) and for ligand-dependent transcriptional activation (Webster et al., 1988; Lees et al., 1989). Estradiol binding induces the formation of receptor dimers, which is essential for recognition of and tight binding to the ERE (Kumar and Chambon, 1988). The poorly conserved C-terminal region (F) can be deleted without affecting hormone binding or transcriptional activation (Kumar, et al., 1987).

The response elements on DNA for the steroid hormones are comprised of inverted repeats with the two half-sites separated by a three nucleotide gap. The consensus sequence of the repeats for the ERE is AGGTCA nnn TGACCT (Ryffel et al., 1988). This was derived from the rat prolactin gene and the vitellogenin gene of Xenopus and of chicken by gene transfer experiments and receptor-DNA binding studies (Klein-Hitpass et al., 1986; Maurer and Notides, 1987; Waterman et al., 1988; Burch et al., 1988.). DNA binding specificity for the nuclear hormone receptors is determined by three residues in the first finger of the zinc region in the DNA binding domain (Mader et al., 1989). The ER contains a glutamate, glycine, and alanine in these three discriminatory positions. Amino acid residues in the second finger appear to determine the appropriate spacing between the half-sites required for receptor binding (Umesono and Evans, 1989).

The palindromic sequence and two-fold rotational symmetry of the response elements suggest that the nuclear

receptors bind to DNA as dimers (Green and Chambon, 1988; Forman and Samuels., 1990). [See figure 3, p. 55.] Each ER molecule would bind one arm of the ERE palindrome. Studies using anti-estrogen receptor monoclonal antibodies (Linstedt, et al., 1986), and direct experimental evidence from DNA binding studies (Kumar and Chambon, 1988), have substantiated this speculation for the ER. Analysis of ER heterodimer formation in a gel shift assay (Kumar and Chambon, 1988) and site-directed mutagenesis studies (Fawell et al, 1990) indicate that dimerization activity is contained within the ligand binding domain (LBD). Estrogen binding is spatially close to or involved in the dimer interface which presents a means for steroid-stabilized dimerization based on hydrophobic shielding (Fawell et al., 1990).

Target gene specificity may be conferred by the variations between different HRE (Forman and Samuels, 1990), by cell or tissue-specific regulation of receptor levels (Strahle et al., 1989), by transcription factors which may be necessary for full receptor activity (Meyer et al, 1989), and/or by tissue specific hormone metabolism (Funder et al., 1988). However, the factors which distinguish positive and negative response elements are not yet known. Lannigan and Notides (1989) found that the ER binds to the dissociated "coding strand" of ERE containing DNA fragments with much greater affinity than to the "noncoding strand" of the ERE

or to the double-stranded ERE. In addition, the dissociated strands of this ERE coding fragment were found to possess unusual secondary structure. Thus, the coding strand of the ERE may fold into a 3-dimensional structure that forms the high-affinity estrogen binding site. Lannigan and Notides proposed that estrogen induces a conformational change in the receptor, which increases its affinity for the double-stranded ERE; hence, the ERE may act initially as a "sink" to concentrate the activated receptor. Transitory strand separation and supercoiling of the ERE permits a transition of the strands into their secondary structure. The ER then preferentially binds to the coding strand and stabilizes the single strand form. Formation of an activated-receptor coding strand ERE complex, then, may be crucial for estrogen-induced transcriptional enhancement. The results of this study also imply that DNA secondary structure might play a more active role in gene transcriptional enhancement than previously thought. Furthermore, the presence of DNA structural elements would provide an additional molecular basis for distinguishing between the steroid receptor response elements which have a high degree of sequence homology.

The mechanism by which estrogen or other ligands in this receptor superfamily activates their respective receptors remains to be clearly established. A trans-activation domain is defined as a portion of the receptor

that, when activated to a DNA binding state, produces an increase in transcriptional initiation (Carson-Jurica et al., 1990). The ligand binding domain (LBD) is thought to inhibit trans-activation. This inhibition is relieved upon hormone binding; deletion of this domain results in a constitutively active receptor (Hollenberg et al., 1987; Godowski, et al., 1987). A region near the N terminus of the LBD termed inactivator of transcription (t_i) is 20-45% conserved among all receptors in the superfamily. Hormone-relieved inactivation can be transferred to the myc oncoprotein (c-myc) simply by fusing this protein to the LBD of the estrogen receptor, which then results in hormone-dependent cell transformation (Eilers et al., 1989).

A great wealth of information has been gathered recently which has helped to elucidate ER-mediated events in peripheral tissues. It appears likely that many of the intervening steps and signal transduction mechanisms may be similar for neural tissues. However, the specific gene targets which play a role in the estrogen-ER initiated cascade of genomic events culminating in CNS sexual differentiation are largely unknown.

Summary and Specific Aims

The development of gender differences in the brain which ultimately results in varied behavioral patterns between the sexes are orchestrated, in part, by an estrogen-

initiated cascade of events during a perinatal "critical period". Estrogen, synthesized intraneuronally from the aromatization of testosterone or carried into the brain from endogenous sources, binds with high affinity to intracellular receptors. The estrogen receptor (ER) is a ligand-activated transcription factor which alters the expression of target genes in hormone sensitive targets. These genomic effects then initiate the neural processes involved in sexual differentiation of the nervous system. Although many of the specific cellular and molecular events mediated by the ER are largely unknown, estrogen has well characterized neurotrophic effects on responsive neural circuitry which may form the basis for gender differences in brain function and behavior.

Numerous anatomical localization and biochemical studies have characterized the topographical distribution and quantity of ER in the adult rodent brain. In addition, studies have described the developmental profiles of the ER in brain sites governing reproductive function and related behaviors, as well as in areas of the cerebral cortex. However, an examination of the development of the ER system has not previously been investigated in the rodent hippocampus.

The evidence for sex differences in hippocampal morphology and function as well as for variations in cognitive behaviors in which the hippocampus plays a key

role suggest that this limbic region is sensitive to the organizational influence of estrogen. Therefore, an examination of the ER system during hippocampal development, when gender differences are established, is potentially important.

The specific aims of the three studies performed for this dissertation are delineated below:

1) To evaluate the postnatal development profile of ER protein in the male and female rat hippocampus and to determine whether the hippocampal ER of the neonate is functional in its ability to be transformed to an activated DNA binding state.

2) To determine if changes in hippocampal ER protein levels during development are due to alterations in ER gene expression by examining steady-state ER mRNA concentrations.

3) To understand the coordinated series of events which provide a basis for estrogen induction of sexual differentiation by examining the mechanisms by which neural ER are regulated during ontogeny. My major objective was to distinguish between the potential intrinsic and epigenetic factors regulating ER expression in the developing hippocampus and other brain regions.

CHAPTER III

TRANSIENT ELEVATION OF ESTROGEN RECEPTORS IN THE NEONATAL RAT HIPPOCAMPUS

Abstract

The presence of sex differences in hippocampal morphology and function suggests that this brain region may be sensitive to the organizational actions of gonadal steroids. We therefore examined the postnatal development of estrogen receptor (ER) in the rat hippocampal formation. ER was measured by the *in vitro* binding of ^{3}H-estradiol to a cytosolic preparation. Radioinert R2858 (moxestrol) was used to determine nonspecific binding. Hippocampal ER concentrations increased from birth through postnatal day (PND) 4 when levels peaked (10.05 $\pm$ 1.2 fmol/mg protein); these were maintained through PND-7 (9.45 $\pm$ 1.4) and declined thereafter to low levels characteristic of the adult (2.05 $\pm$ 0.35). This ontogenic profile is similar to that found in several neocortical regions, as well as in the cingulate cortex, but is distinct from that observed in the hypothalamus, where ER levels remain high in the adult. Saturation analysis of PND-7 hippocampal cytosols demonstrated a single, high affinity binding site (Kd: 5.51 $\pm$ 1.7 x 10^{-10} M). ^{3}H-estradiol binding was specific in that

it was displaced by radioinert R2858, diethylstilbestrol (DES), and 17-beta estradiol but not by nonestrogenic steroids. Significantly greater ER levels were found in hippocampal nuclear extracts from DES-treated PND-7 animals compared to controls (9.74 ± 2.27 vs. 0.49 ± 0.24 fmol/mg DNA; $p < .01$). The presence of functional ER was also shown by the ability of receptors to be retained on DNA cellulose. DNA cellulose column chromatography elution profiles for PND-7 hippocampal and medial basal hypothalamic (MBH) cytosols following incubation with ^{3}H-estradiol were similar. The presence of elevated hippocampal ER levels during the perinatal critical period and evidence of functional transformation to the DNA binding state following DES treatment *in vivo* or estrogen incubation *in vitro* suggests that the hippocampus is a potential substrate for estrogen-mediated organizational events.

Introduction

Sexual differentiation of the central nervous system (CNS) in most mammalian species results from gender differences in gonadal steroid hormone secretion during a critical developmental period (MacLusky and Naftolin, 1981; McEwen, 1983; Toran-Allerand, 1984). Males are exposed perinatally to testosterone while females are not (Pang et al., 1979; Weiscz and Ward, 1980). The intracellular aromatization of testosterone to 17-beta-estradiol and its

subsequent binding to high affinity estrogen receptors appears to be paramount for the male specific organization of many neural structures and functions (Toran-Allerand, 1984). A subsequent cascade of molecular and biochemical events mediates the maturational processes which occur perinatally to organize brain structure and function into the male phenotype.

The hippocampal formation exhibits sex specific differences in several morphological (Diamond et al., 1982, 1983; Wimer and Wimer, 1985; Wimer et al., 1988), neurochemical (Harrelson and McEwen, 1987; Loy and Sheldon, 1987; Shephard et al., 1982; Turner and Weaver, 1985), and electrophysiological attributes (Loy, 1986; Teyler et al., 1980). In the adult hippocampus, estrogen influences certain neurotransmitter/ neuropeptide systems (Badr et al., 1988; Beck et al., 1990; Maggi and Perez, 1984; Perez et al., 1988) which may occur through a genomic mechanism as suggested by studies demonstrating enhanced mRNA production following estrogen exposure (Maggi et al., 1989; Perez et al., 1988). The hippocampus mediates several adult behaviors and functions which are sexually dimorphic. For example, the hippocampus has been strongly implicated as a substrate for learning and memory functions and spatial mapping of the animal's environment (Friedman and Goldman-Rakic, 1988; Mahut and Moss, 1984; O'Keefe and Nadel, 1978; Sutherland et al., 1982; Zola-Morgan and Squire, 1986).

studies performed in many species, including humans, have shown that males demonstrate superior performance in tasks involving spatial abilities and their acquisition (Beatty, 1984). Gender-related differences in hippocampal dendritic plasticity during development (Juraska et al., 1985, 1989) and on axonal reorganization following partial deafferentation in adult animals (Loy and Milner, 1980; Milner and Loy, 1980; Morse et al., 1986) have also been reported. Many of these sexually differentiated functions/responses are dependent upon early exposure of the CNS to gonadal steroids (Joseph et al., 1978; Milner and Loy, 1982). This implies that the receptor(s) for the steroid hormone(s) responsible for hippocampal sexual differentiation should be present during the critical period for such organizational events.

The existence of low levels of estrogen receptors has been shown in the dentate gyrus, pyramidal cell layer of Ammon's horn, and subiculum of the adult rat (Loy et al., 1988; Pfaff and Keiner, 1973; Rainbow et al., 1982). Recent evidence utilizing immunoenzymatic (Maggi et al., 1989) and *in situ* hybridization (Pelletier et al., 1988) methodologies suggests that high levels of the estrogen receptor protein and its mRNA are present in the hippocampus of adult rats. However, the ontogeny of estrogen receptors in the hippocampal formation has heretofore not been investigated. Previous studies have demonstrated that gonadal steroid

receptor concentrations observed in neonates may be quantitatively different from those seen in adult animals (Friedman et al., 1983; Gerlach et al., 1983; Handa et al., 1988; Keefer and Holderegger, 1985; MacLusky et al., 1979; Shughrue et al., 1990; Vito and Fox, 1982). In addition, numerous hypothalamic studies indicate that the rodent brain exhibits heightened sensitivity to the organizational influence of gonadal steroids during the first postnatal week (Arnold and Breedlove, 1985). We have therefore utilized quantitative *in vitro* receptor binding assays to examine the postnatal developmental profile of estrogen receptors in the rat hippocampus.

Materials and Methods

Animals

Neonatal rats of various ages from timed-pregnant Sprague-Dawley females and male and female adult rats were used in these studies (Sasco; Omaha, NE). Animals were maintained on a 12:12 hour light/dark cycle (lights on at 0700). Food and water were available ad libitum. On the day of parturition pups were culled to 8-10 per litter. Animals were sacrificed at postnatal days (PND)-0, 1, 4, 7, 10, 13, and 17 and at 2-4 months (adults). The first postnatal week was particularly emphasized as this period has been shown to be critical for the organizational effects of gonadal steroids on other sexually dimorphic brain structures and functions (Arnold and Breedlove, 1985).

postnatal days were calculated using the day of birth as PND-0. All animals (except those used in the DNA cellulose affinity column chromatography experiments) were bilaterally gonadectomized 12-24 hours prior to sacrifice to remove endogenous gonadal steroids which could interfere with *in vitro* ligand binding assays and to recycle nuclear bound receptor to the cytosolic form. Surgery was performed under hypothermic anesthesia for PND-1 to PND-4 animals and under ether anesthesia at later ages. PND-0 animals were not gonadectomized prior to receptor binding assays.

Tissue dissection

Animals were killed by decapitation and the brains were immediately removed and placed on ice (adult animals) or in ice-cold TEGMD buffer (10 mM Tris-HCL, 1.5 mM EDTA, 10% glycerol, 25mM molybdate, and 1mM dithiothreitol, pH 7.4) (neonatal animals). Tissues from 3 (PND-0 and PND-1) or 2 (all other age groups) animals of the same sex were pooled for each determination. The following brain regions were block dissected on an ice-cold brass plate with the brain placed ventral side up: entire dorsal and ventral hippocampal formation (subicular complex, Ammon's horn, and the dentate gyrus), medial basal hypothalamus (MBH), and cerebral cortex [medial frontal cortex (MFC), frontal cortex (FC), cingulate cortex, and parietal cortex]. The MBH was removed as a wedge of tissue from a section made just

posterior to the optic chiasm and anterior to the mammillary bodies using the hypothalamic sulci as lateral boundaries and the top of the third ventricle as the dorsal boundary. In addition, slices of parietal cortex were removed from this section. The entire MFC (included cingulate areas 1,2 and infralimbic regions) was removed bilaterally as slices of tissue anterior to the corpus callosum. The FC consisted of the remaining cortical tissue (frontal areas 1-3) from the section anterior to the corpus callosum after the olfactory bulbs were removed. The cingulate cortex was that region dorsal to the corpus callosum, 1 mm from the midline and included the cingulate 1-3 and retrosplenial areas. The dissection coordinates were consistent with the atlas of Paxinos and Watson (1986).

Cytosolic estrogen receptor (ER) binding assay

Cytosolic ER content was quantified using a modification of methods described by MacLusky and McEwen (1979). Tissue samples were homogenized in 300 *u*l of ice-cold TEGMD buffer in Dounce tissue grinders (Wheaton Scientific, Millville, NJ). The homogenates were transferred to 9 X 50 mm polyethylene tubes and centrifuged at 800 x g in a Beckman TJ-6R centrifuge (Beckman Instruments, Palo Alto, CA) for 10 minutes at 4°C. The resulting nuclear pellet was stored at -70°C until DNA content was quantified by the method of Burton (1956), using

calf thymus DNA as the standard. The supernatants were decanted and recentrifuged at 105,000 x g for 15 minutes at 4°C in a Sorvall OTD 55B ultracentrifuge (Dupont, Wilmington,DA). One hundred ul aliquots of the supernatant cytosols were incubated with 4nM [^{3}H]-estradiol 17-beta (New England Nuclear, Wilmington,DE; 96.4 Ci/mmol) for 4 hours at 25°C in a total incubation volume of 150 ul. Parallel incubations containing a 200 fold excess (.8 uM) of radioinert moxestrol (R2858; New England Nuclear, Wilmington, DE) in addition to [^{3}H]-estradiol were used to assess nonspecific binding. R2858 was used since it does not bind to alpha fetoprotein or other plasma proteins but displays a high affinity for the ER (McEwen et al., 1975). Incubates were cooled on ice for 10-15 minutes following the incubation period. Bound radioactivity was separated from free radioligand by Sephadex LH-20 column chromatography. One hundred thirty five ul of each incubate were applied to miniature columns of Sephadex LH-20 (Sigma Chemical, St. Louis, MO) equilibrated with TEGMD buffer at 4°C. Columns were made from 1 ml Pipetman (Labcraft, Curtin Matheson Scientific, Inc., Houston, TX) pipette tips (bed height 5.9 cm; diameter, 8 mm). Samples were washed into the columns with 100 ul TEGMD buffer. Flow through the columns was allowed to stop for 30 minutes and the macromolecular bound [^{3}H]-estradiol was eluted with 600 ul TEGMD buffer directly into polyethylene liquid scintillation vials. Three ml of

Ecoscint scintillation fluid (National Diagnostics) was added and radioactivity was counted on a Beckman LS7000 scintillation counter (Beckman Instruments, Palo Alto, CA) to less than 5% error at 37% counting efficiency. Specific binding was determined by subtracting nonspecific binding from total counts bound. Data have been expressed as femtomoles bound per mg protein and per mg DNA. Protein content was determined by the method of Lowry, et al.(1951) using bovine serum albumin as the standard.

Saturation Analysis

Saturation analysis of [^{3}H]-estradiol binding was determined in the hippocampus, combined MBH and preoptic area (HPOA),or cerebral cortex (pooled medial frontal, frontal, cingulate, and parietal cortices) of PND-7 and adult animals. The preoptic area was removed from a section made just anterior and posterior to the optic chiasm using the anterior commissure as its dorsal limit and extending 1 mm to either side of the third ventricle. Tissues from 9-10 (PND-7) or 6-8 (adult) rats of the same sex were pooled and homogenized in TEGMD buffer. The cytosolic fractions were prepared as previously described. Cytosols were incubated with increasing amounts of [^{3}H]-estradiol (0.1 to 5nM). Parallel incubation tubes contained 1uM radioinert R2858 to determine nonspecific binding. Data were analyzed with a computerized, statistically weighted regression analysis

program, LIGAND (Munson and Rodbard, 1980).

^{3}H-estradiol binding specificity

In another series of experiments, the specificity of ^{3}H-estradiol binding was examined in the hippocampus and HPOA of PND-7 animals. Animals were gonadectomized under ether anesthesia 24 hours prior to sacrifice. Tissues from 9 or 10 animals of the same sex were pooled and homogenized in TEGMD buffer and cytosols were prepared as described above. Competition analysis was performed by incubating the cytosolic fractions with 1 nM [^{3}H]-estradiol in the presence of varying concentrations of radioinert R2858 (0.1 to 200 nM) or with 10 nM radioinert 17 beta-estradiol (E), diethylstilbestrol (DES), progesterone (P), testosterone (T), dihydrotestosterone (DHT), corticosterone (CORT), or dexamethasone (DEX) (Sigma Chemical, St. Louis, MO). [^{3}H]-estradiol binding in the presence of radioinert steroids was then compared to that found in the absence of such compounds (control incubation tubes).

Nuclear ER exchange assay

The question of whether hippocampal ER in the neonate could be transformed *in vivo* was examined via a modification of the cell nuclear ER exchange assay described by MacLusky and McEwen (1979). PND-7 animals were injected subcutaneously with 2 ug DES dissolved in 100 ul of a 5%

ethanol/saline solution or vehicle. Animals were sacrificed by decapitation 60 to 90 minutes later, and their brains dissected. DES was injected as it possesses a high affinity for the ER but binds minimally to the plasma protein, alpha-fetoprotein (Sheehan and Young, 1979). Tissues were homogenized in 300 ul of Buffer A (1mM KH_2PO_4, 3mM $MgCl_2$, 10% glycerol, 0.32 M sucrose, 1 mM dithiothreitol, pH 6.8) containing 0.25% Triton X-100. The homogenates were centrifuged at 800 x g in a Beckman TJ-6R centrifuge at 4°C for 10 minutes. The supernatant fraction was removed and discarded. The pellets were resuspended in 25 ulof buffer A containing 5 mg cellulose fibers (Sigmacell, Sigma Chemical, St. Loius, MO). The cellulose was used to mark the pellet following centrifugation. It was washed in ethanol, distilled water(twice), and buffer A prior to use to remove residual pyridines. One hundred ten ulof Buffer B (1 mM KH_2PO_4, 0.32 M sucrose, 1 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, pH 6.8) was added to the suspension and mixed. Purified nuclear preparations were obtained by centrifugation at 50,000 x g for 20 min. at 4°C. The pellicle and sucrose buffers were then removed and the walls of the tube were carefully wiped dry with a cotton tipped applicator. The pellet was suspended in 110 ulof TEBD buffer (10 mM Tris, 1.5 mM EDTA, 0.5 mM bacitracin, and 1.0 mM dithiothreitol, pH 7.4) and after 5 minutes an additional 110 ulof TEBDK buffer (TEBD buffer + 0.8 M KCL)

was added to achieve a final salt concentration of 0.4 M. The contents of each tube were mixed intermittently for 25 minutes. The salt extract was separated from cellex and nuclei by centrifugation at 25,000 x g for 5 minutes and the supernatant was used to assay nuclear ER. One hundred ulaliquots of the salt extracts were incubated for 4 hours at 25°C with 4nM ^{3}H-estradiol in TEBD buffer (total incubation volume -150 ul). A parallel series of incubation tubes to correct for nonspecific binding contained 200 fold excess (.8uM) radioinert R2858 in addition to [^{3}H]-estradiol. Incubates were cooled for 10-15 minutes on ice following the incubation. Bound and free steroid were separated by Sephadex LH-20 column chromatography. Radioactivity was counted in 3ml of scintillation fluid (Ecoscint) on a 1900 CA TRI-CARB liquid scintillation analyzer (Packard Instruments, Meriden, CT) at 39% counting efficiency. All data were expressed as fmol bound/mg DNA.

DNA Cellulose Affinity Column Chromatography

DNA cellulose column chromatography was utilized to examine whether hippocampal ER are functionally able to transform to the DNA binding state following incubation with ligand. PND-7 intact female rats were utilized in these experiments. Animals were sacrificed by decapitation and their brains removed and dissected to the hippocampus and MBH. Tissues from 7 animals were pooled and homogenized in

TEG buffer (10 mM TRIS, 1.5 mM EDTA, 10 % glycerol, 1 mM monothioglycerol, pH 7.4). The homogenates were centrifuged at 106,000 X g for 20 minutes. The supernatant cytosolic fractions were removed and aliquots assayed for protein concentration via the method of Lowry, et al.(1951). One hundred ulof cytosols were incubated with 4nM [^{3}H]-estradiol in TEG buffer (total incubation volume - 150 ul) at 0 - 4 °C for 1 hour. Nonspecific binding tubes also contained a 200 fold excess (0.8 uM) of radioinert R2858 in addition to [^{3}H]-estradiol. The incubates were applied to columns of single-stranded calf thymus DNA cellulose (Sigma Chemical, St. Louis, MO) previously equilibrated with TEG buffer. The columns were prerinsed with a whole brain cytosol preparation and radioactivity (4nM - ^{3}H-estradiol) as estradiol binds nonspecifically to DNA cellulose. The columns were then rinsed with TEG + 0.5 M KCL to strip them of any radioactivity, DNA binding proteins, or loose DNA. The columns were re-equilibrated with TEG buffer, cytosols were applied to the columns, allowed to enter the columns by gravity flow and rinsed into column with 100 ulof TEG buffer. Flow through the columns was stopped and the cytosols were allowed to incubate on the columns for 1 hour at 25°C. The columns were allowed to cool for 30 minutes at 4°C and unbound radioactivity was then eluted with 36 ml of TEG buffer. The hormone-bound ER which adhered to the DNA-cellulose were eluted with an increasing stepwise gradient

of TEG + KCL (from 0.0 to 0.3 M KCL). One ml fractions were collected and radioactivity was counted following the addition of scintillation fluid.

Statistics

Differences in cytosolic ER concentrations across time in the same brain region were analyzed via a two way analysis of variance (ANOVA) with sex and age as the two factors. Additionally, a two way ANOVA was used to assess differences in nuclear ER numbers between DES- and vehicle-treated groups with sex and treatment as the two factors.

Results

Developmental profile of cytosolic ER in hippocampus, cerebral cortex, and MBH

Specific [^{3}H]-estradiol binding in cytosolic fractions of hippocampus, other cerebral cortical regions, and the MBH during postnatal development, expressed in fmol/mg cytosolic protein, is illustrated in figure 1. No significant sex differences in ER concentrations in the above brain regions were found at any of the ages examined (two way ANOVA). Therefore, data from males and females were combined in all groups. Hippocampal ER concentrations increase from PND-0 through PND-4 when the highest concentration was found (10.05 $\pm$ 1.2 fmol/mg protein) . These were maintained through PND-7 and declined thereafter to reach low adult levels (2.05 $\pm$ 0.35 fmol/mg protein). A similar ontogenic profile was found in the medial frontal, frontal, parietal, and cingulate cortices (data not shown). However, in the MBH, ER concentrations increase through the second postnatal week to reach adult levels (25-30 fmol/mg protein). A similar pattern was observed when ER are expressed as fmol/mg DNA (data not shown). At PND-13, decreases in ER levels from PND-7 values are seen in the hippocampus and other cortical regions, whereas ER continue to increase in the MBH.

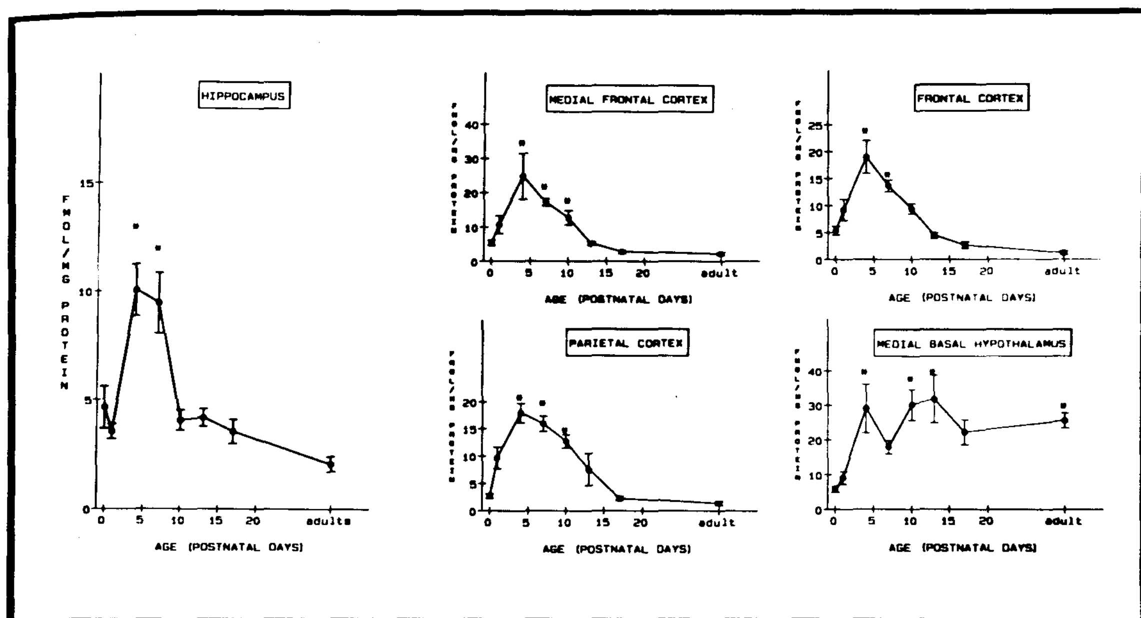


FIGURE 1.

Cytosolic [3H]-estradiol binding (mean + S.E.M.; fmol/mg protein) in various brain regions on postnatal day (PND) 0 (n= 10-12), 1 (n= 4-6), 4 (n=5-12), 7 (n= 11-13), 10 (n=11-13), 13 (n= 11-13), 17 (n=6-9), and in adult animals (n= 5-9). Tissues from 3 (PND-0 and PND-1) or 2 (all other age groups) animals of the same sex were pooled for each determination. No significant sex differences in ER concentration were found at any age in all brain regions; therefore data from males and females were combined in all groups. * designates those points which are significantly different ($p < 0.05$) from PND-0 levels [Two way ANOVA/Tukey-Kramer multiple pairwise comparisons test].

Saturation isotherms of cytosolic [^{3}H]-estradiol binding in the hippocampus, HPOA (Fig. 2), and pooled frontal, parietal and cingulate cortices (data not shown) of PND-7 and adult animals were constructed. Statistically weighted linear regression analysis using the LIGAND program yielded highly significant correlation coefficients (r= -0.91 to 0.98) with apparent dissociation constants (K_d) of 5.51 $\pm$ 1.7 x 10 $^{-10}$ M (n=4) for the hippocampus, 1.21 $\pm$ 0.43 x 10^{-10} M (n=4) for the HPOA, and 7.99 x 10^{-10} M (n=2) for the pooled cortices.

Specificity of [^{3}H]-estradiol binding in cytosolic extracts was determined by competition with radioinert R2858 in concentrations ranging from 0.1 to 200 nM and radioinert 17 beta-estradiol (E_2), DES, P, T, DHT, CORT, and DEX at 10nM concentrations (10 fold excess) in the hippocampus and HPOA of gonadectomized PND-7 animals (Fig. 3). No sex differences were found in the ability of the various radioinert steroids to compete for ^{3}H-[E2] binding in either brain region. Therefore, values obtained from males and females were combined for analysis. R2858, E_2, and DES were potent competitors of [^{3}H]-estradiol binding to hippocampal and HPOA cytosols. R2858 demonstrated an IC_{50} of approximately 0.2 nM. None of the other steroids were able to compete effectively for [^{3}H]- estradiol binding sites.

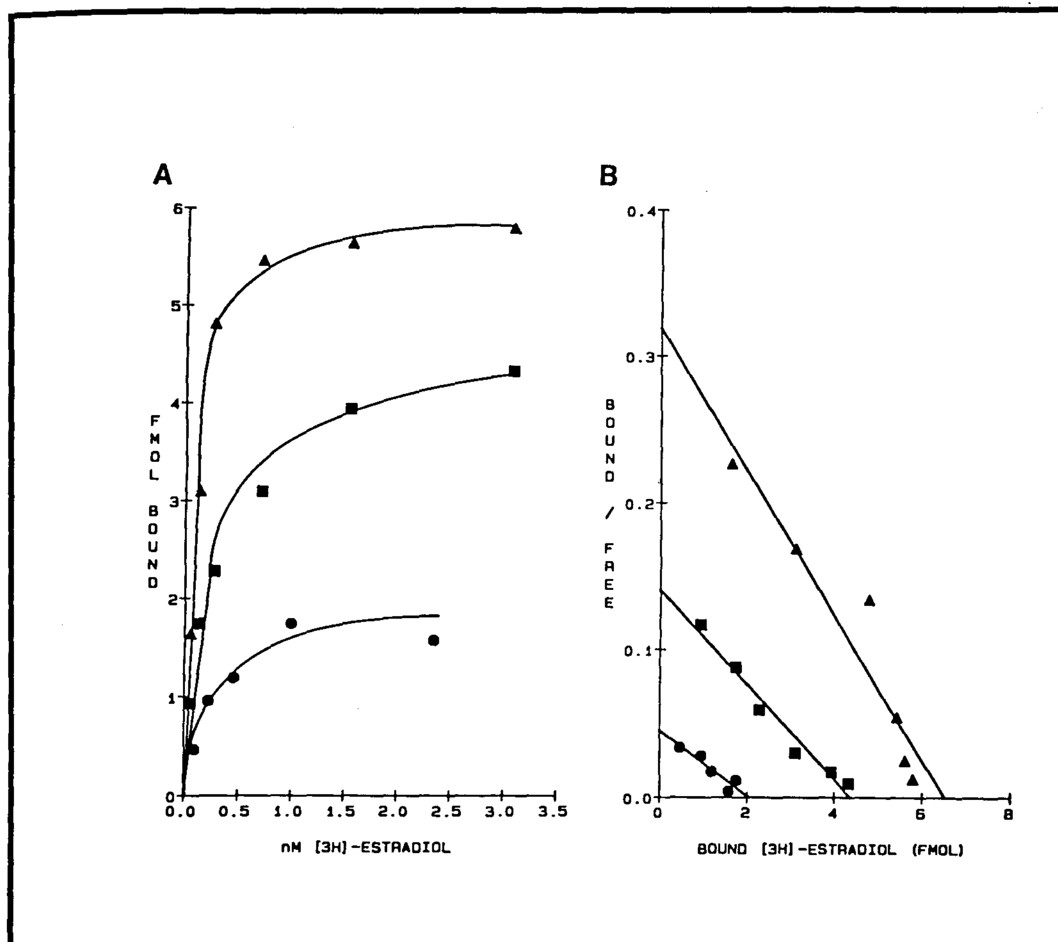


FIGURE 2.

Representative saturation isotherm (A) and Scatchard plot (B) for specific [3H]-estradiol binding in cytosolic extracts from PND-7 hippocampus (HIP) [square], PND-7 hypothalamus/preoptic area (HPOA) [triangle], and in adult HIP [circle]. Kd values were 2.22 x 10-10M, 1.44 x 10-10M, and 3.19 x 10-10M, respectively. Bmax values were 7.11 fmol/mg protein for the PND-7 hippocampus, 34.89 fmol/mg protein for the HPOA, and 2.71 fmol/mg protein for the adult hippocampus. Tissues from 9-10 (PND-7) or 6-8 (adult) animals of the same sex were pooled for the analysis.

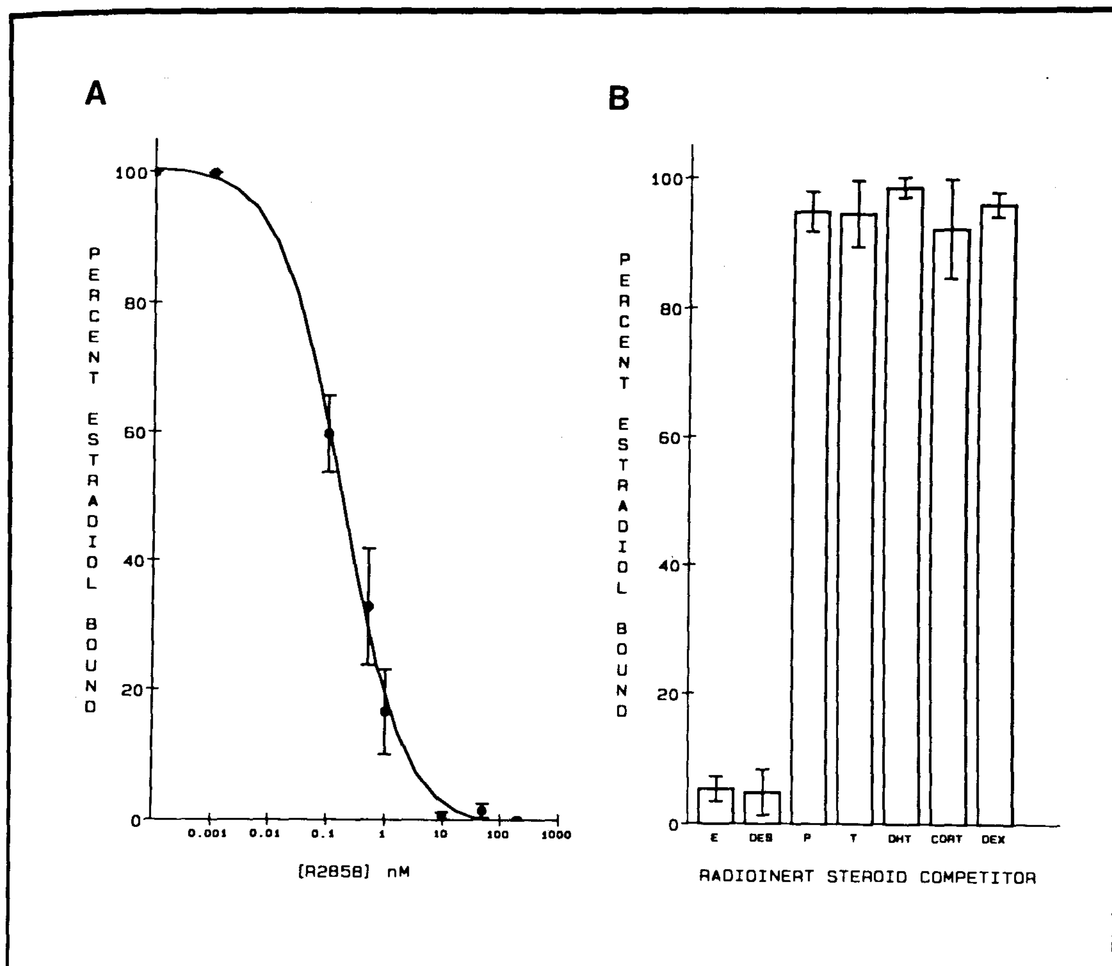


FIGURE 3.

Competition for [3H]-estradiol binding to PND-7 hippocampal cytosols by radioinert steroids. Each point represents the mean ± S.E.M. (n= 4) of the percent total [3H]-estradiol bound in the presence of competing steroid. Tissues from 9-10 animals of the same sex were pooled for each determination. No sex differences were found in the competitive ability of the radioinert steroids; therefore values obtained from males and females were combined for analysis. R2858 was used in concentrations ranging from 0.1 to 200 nM (A) and various radioinert steroids were used at a 10 nM concentration (B). E, 17 *B*-estradiol; DES, diethylstilbestrol; P, progesterone; T, testosterone; DHT, dihydrotestosterone; CORT, corticosterone; DEX, dexamethasone.

Following the administration of DES to gonadectomized rats, a significantly greater concentration of ER are found in hippocampal nuclear extracts as compared to vehicle-treated controls ($p < .01$) (Fig. 4). No significant sex differences were found in the number of nuclear ER in either the DES or vehicle groups (two way ANOVA); therefore values from males and females were pooled in the analysis. Elevated ER levels were also found in the MFC, PFC, and MBH following DES treatment (data not shown). DNA-cellulose affinity chromatography elution profiles were similar for the hippocampus and MBH on PND-7 (Fig. 5). DNA-bound receptor was eluted from the columns with 0.2 to 0.3 M KCL. The control incubation tubes containing radioinert R2858 in addition to [^{3}H]-estradiol gave no detectable radioactivity eluting in the 0.2 to 0.3 region of the chromatograms.

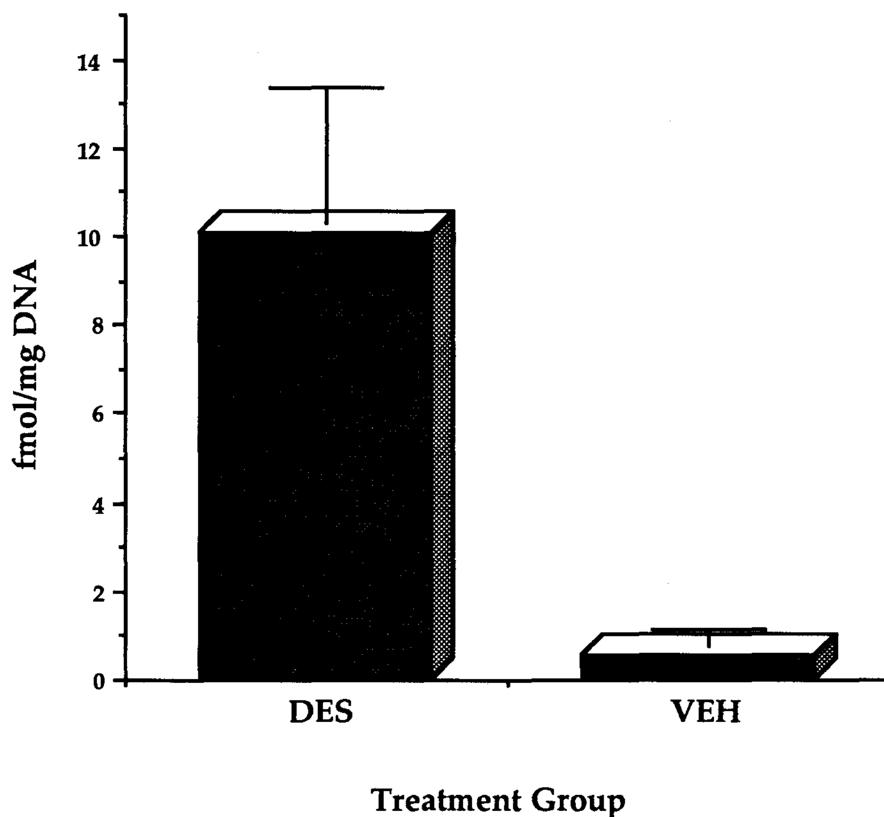


FIGURE 4

[3H]-estradiol binding to nuclear extracts from hippocampus of diethylstilbestrol (DES)- and vehicle (VEH)- treated PND-7 male and female rats. No significant sex differences were found in nuclear binding in either group and therefore values from males and females were pooled for the illustration. Each point represents the mean ± S.E.M. of 9-11 determinations. * $p < 0.01$; Two way ANOVA.

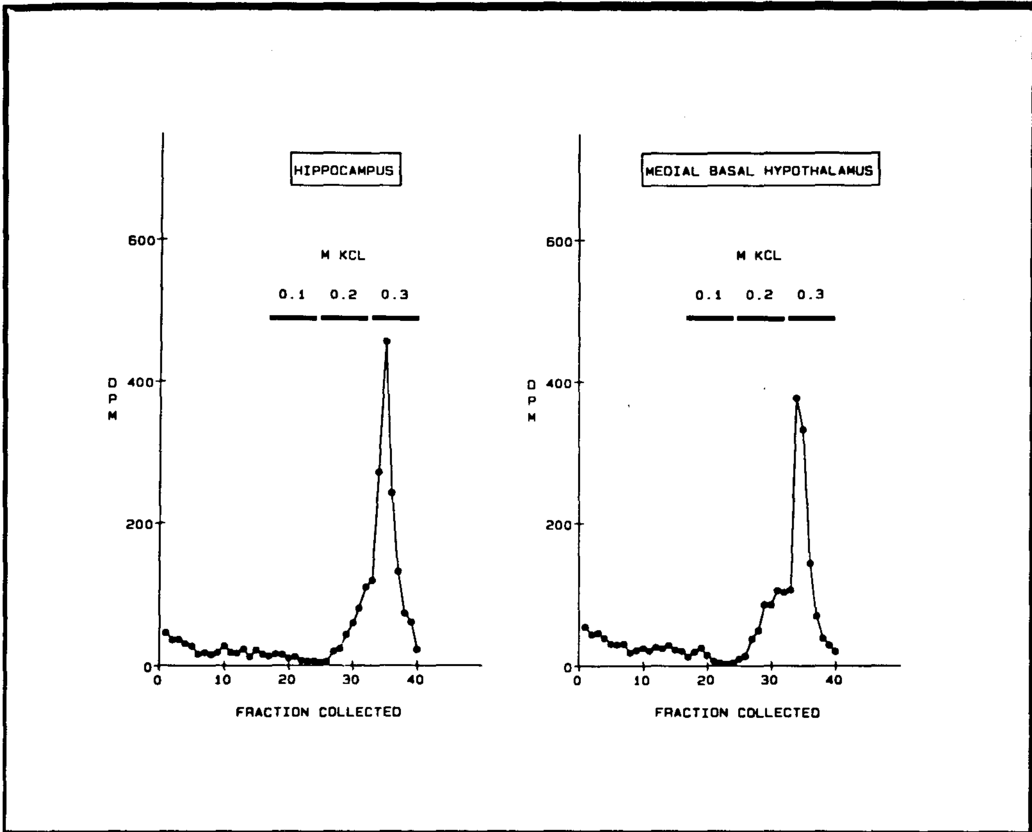


FIGURE 5.

DNA-cellulose affinity chromatography elution profiles for [3H]-estradiol binding to cytosols from the hippocampus and medial basal hypothalamus on PND-7 (pooled tissues from 7 females). Nonspecific binding was approximately 5-15 DPM/fraction. Hormone-bound ER adhering to DNA cellulose were eluted with an increasing stepwise gradient of TEG + KCl. One ml fractions were collected throughout.

Discussion

These studies demonstrate a transient elevation of ER in the rat hippocampus during the early postnatal period. The receptors are highly specific for natural and synthetic estrogens and represent a single, high affinity binding site for estradiol. These binding characteristics suggest that hippocampal ER of the neonatal rat are identical to those found in other regions of the CNS.

Our data showing low ER levels in the hippocampus of the adult rat are in agreement with previous studies that utilized both autoradiographic (Loy et al., 1988; Pfaff and Keiner, 1973), biochemical (Rainbow et al., 1982), and immunocytochemical (Cintra et al., 1986) methods to assess ER concentration. However, recent studies using immunochemical techniques detected high ER levels in the hippocampus of adult female rats (Maggi et al., 1989) and variable levels in the murine female hippocampus depending on circulating estrogen levels (Koch and Ehret, 1989). The reasons for these discrepancies are unclear but may reflect species-specific variation and/or methodological differences. A report of high ER mRNA levels detected by *in situ* hybridization in the hippocampus (and other cerebral cortical regions) of the adult rat (Pelletier et al., 1988) may support those studies reporting high levels of ER protein in the hippocampal formation (Koch and Ehret, 1989; Maggi et al., 1989). Alternatively, elevated ER mRNA

without coincident ER levels could suggest altered posttranscriptional processing of ER mRNA in the adult and/or the development of receptor degrading enzymes following the early postnatal period.

The developmental profile of ER levels in the hippocampus is strikingly similar to that observed in various neocortical regions and in the cingulate cortex. Earlier studies in both the rat and mouse have also demonstrated a peak in the amount of cortical ER at the end of the first week of life; levels decline thereafter to reach low values (Friedman et al, 1983; Gerlach et al., 1983; MacLusky et al., 1979). Our data indicates that the peak in ER levels occurs slightly earlier (by PND-4) in the hippocampus and other cortical regions than that previously reported in the mouse cingulate and neocortex. However, these levels are maintained through PND-7 before beginning their descent. The ontogenic profile of ER in the neocortex and hippocampus contrasts with that observed in the hypothalamus, where ER concentrations increase during the perinatal period but remain high in the adult (Friedman et al., 1983; Gerlach et al., 1983; Vito and Fox, 1982).

The function of elevated ER in the developing nervous system is not presently understood. The fact that the developmental profile of ER is virtually identical in all regions of the cerebral cortex examined thus far (Friedman et al., 1983; Keefer and Holregger, 1985; MacLusky et al.,

1979), including allocortical regions such as the hippocampus and cingulate cortex, may indicate that ER ontogeny and function is a component of a general cortical maturation process. The peak in ER levels during the first postnatal week occurs in a milieu of rapid neuronal growth and differentiation (Bayer, 1980). Interestingly, certain regions of the hippocampus, such as the dentate gyrus, develop later than other cerebral cortical regions, but this does not seem to influence the development of ER in our preparation using the entire hippocampus. Estrogen administration during the early postnatal period has been shown to enhance cortical myelination (Curry and Heim, 1966), a finding which supports a developmental role for estrogen in maturation of the cerebral cortex.

The presence of high ER levels in the hippocampus during a period when other sexually dimorphic brain regions demonstrate heightened sensitivity to gonadal steroids (Arnold and Breedlove, 1985) suggests that the ER may be important for steroid-mediated organization of this brain region. Estrogen has been shown to enhance neurite outgrowth from dissociated cerebral cortical cell cultures (Uchibori and Kawashima, 1985) and from ER-containing regions of cortical explant cultures (Toran-Allerand, 1984; Toran-Allerand et al., 1988) in a manner similar to that observed in sexually dimorphic hypothalamic/preoptic area regions (Toran-Allerand et al., 1980). The behavioral

masculinization on cerebral lateralization tasks seen in prenatally DES-exposed females (Hines and Shipley, 1984) suggests that estrogen may be involved in functional sexual differentiation of the cerebral cortex.

Several possibilities exist as to why hippocampal (and other cerebral cortical) ER numbers decrease after PND-7. An increase in cell numbers and presumably in protein mass with ongoing development could cause an apparent reduction in ER numbers. This may be the case as neurogenesis in the dentate gyrus granule cells continues well beyond the first postnatal week (Bayer, 1980; Schlessinger et al., 1975) and ER have not been localized in this cell type in juvenile rats (Loy et al., 1988). Conversely, there might be selective cell death in ER containing neurons or repression of the ER gene with development. The fact that a similar transient increase in ER content is present when the data are calculated on a per mg DNA basis supports the latter possibility.

Our demonstration of significantly elevated amounts of ER in hippocampal nuclear fractions following DES injection suggests that the receptors are functional in relation to their ability to bind estrogens and transform to the DNA binding state. Moreover, the DNA-cellulose affinity chromatography elution profiles for the PND-7 hippocampus are virtually identical with those observed in the MBH. Whether or not hippocampal ER in the neonate are occupied by

endogenous estrogens remains unclear. Nuclear ER were not detectable in neocortical tissues of early postnatal, intact female rats (MacLusky et al., 1979). This finding may be attributable to the low levels of ovarian hormone secretion at this developmental stage (Pang et al., 1978) and/or the sensitivity of the assay used. Male rats were not assessed for the presence of nuclear ER in that study. One might expect to find nuclear receptors in this population given that serum testosterone levels are higher in males than in females (Weiscz and Ward, 1980) and thus might contribute to the intraneuronal pool of estradiol derived from the aromatization of testosterone. While aromatase activity was not detectable in the dentate gyrus and CA1 field of the hippocampus or in the cerebral cortex of the adult rat (Roselli et al., 1985), significant levels of enzyme activity have recently been reported in hippocampal homogenates of PND-3 to PND-10 rats (MacLusky et al., 1987). Therefore, local estradiol synthesis in the hippocampus of neonatal rats, and subsequent binding to ER contained therein may play a physiologically relevant role in sexual differentiation of this brain region. Testosterone administration in neonatal rats has been shown to accelerate neurogenesis in the dentate gyrus (Seress, 1978). In addition, reports of a greater granule cell size and density and reduced naturally occurring neuronal cell death in the dentate gyrus of male mice as compared to females (Wimer and

Wimer, 1985; Wimer et al., 1988), lends credence to the postulated "neuronotrophic" role for gonadal steroids (Toran-Allerand, 1984) in hippocampal development.

The presence of elevated hippocampal ER during the critical period for sexual differentiation and evidence for their functional transformation to the DNA binding state suggest that the hippocampus is a substrate for estrogen's organizational actions. Our data support the theory that sex differences in cognitive function result from estrogen-mediated developmental mechanisms in a manner similar to those causing differentiation of the hypothalamus. Further studies delineating the ER-containing cell types within the neonatal hippocampus and the demonstration of a biochemical consequence of estrogen action during the development of this brain region are necessary to elucidate the role of sex steroids in hippocampal function.

CHAPTER IV

QUANTITATION OF ESTROGEN RECEPTOR MESSENGER RIBONUCLEIC ACID LEVELS DURING HIPPOCAMPAL DEVELOPMENT

Abstract

We previously reported transiently elevated ER protein levels in the postnatal rat hippocampus suggesting that this brain region may be sensitive to estrogenic trophic and organizational influences during a "critical period" of sexual differentiation. In order to examine whether alterations in ER gene expression underlie the ontogenetic pattern of the hippocampal ER, we examined steady state ER mRNA levels over the early postnatal period and in adult male rats. ER mRNA was measured with a highly sensitive RNAase protection assay allowing us to absolutely quantify message levels. Hippocampal ER mRNA levels increased significantly ($p < 0.005$) between birth and postnatal day (PND) 4 when peak concentrations were found (0.1848 ± 0.014 fmoles/mg total RNA) and declined by PND-10 (0.1206 ± 0.017). Adult male hippocampal ER mRNA values were similar to those found in newborn and PND-10 animals but were significantly less ($p < 0.05$) than those observed on PND-4. Thus, the temporal pattern in steady state ER mRNA levels in the hippocampus correlated well with our previous

developmental profile of the ER protein suggesting that hippocampal ER ontogeny is regulated by alterations in ER gene expression.

Introduction

The hippocampal formation is a limbic cortical structure which subserves several important neural functions including the cognitive processes of learning and memory (reviewed in Alkon et al., 1991) and modulation of the hormonal response to stress through glucocorticoid negative feedback mechanisms (Jacobson and Sapolsky, 1991). In humans, the hippocampus is the principle site of neurodegeneration in Alzheimer's disease (Rogers and Morrison, 1985) and dysfunction in temporal lobe epilepsy (Gloor et al., 1982).

In rodents, mounting evidence over the last decade indicates that the hippocampus is sexually dimorphic in its morphology and function (Pfaff, 1966; Diamond et al., 1982, 1983; Wimer and Wimer, 1985; Loy, 1986; Jurasksa et al., 1985, 1989) and that estrogen affects the growth, differentiation, and plasticity of hippocampal neurons (Morse et al., 1986; Blanco et al., 1990; Gould et al., 1990; Woolley et al., 1990). In addition, some animal and human behaviors in which the hippocampus plays a key role, such as spatial navigation (Morris et al., 1982; Muller and Kubie, 1987), are different between males and females (Dawson et al., 1975; Joseph et al., 1978; Beatty, 1984; Van

Haaren et al., 1990; Hines, 1990). Development or organization of neural gender differences is orchestrated, in part, by an estrogen-initiated cascade of events during the perinatal period (MacLusky and Naftolin, 1981; McEwen 1983). Estrogen, synthesized intraneuronally from the aromatization of testosterone (MacLusky et al., 1987), or carried into the brain from endogenous sources (Toran-Allerand, 1984, 1987), binds to intracellular nuclear estrogen receptors (ER) which act as ligand-activated transcription factors to alter the expression of target genes (Yamamoto, 1985; Carson-Jurica et al., 1990).

In the CNS of rodents, the ER protein and its mRNA are particularly abundant in the preoptic area, the ventromedial and arcuate hypothalamic nuclei and the corticomedial amygdala (Pfaff and Keiner, 1973; Stumpf et al., 1975; Simerly, 1989) where they are intimately involved in the neuroendocrine control of reproduction (Davis et al., 1982; Pfaff and Schwartz-Giblin, 1988; Sachs and Meisel, 1988). However, the rather extensive distribution of ER mRNA recently observed in the adult rat brain suggested that estrogen may modulate a wide variety of neural functions (Simerly et al., 1989). In addition, immature neurons in the developing cerebral cortex and hippocampus of the rodent and primate brain express the ER protein in relatively high amounts (MacLusky et al., 1979; MacLusky et al., 1986; Shughrue et al., 1990; O'Keefe and Handa, 1990; Sibug et

al., 1991). These findings provide a potential cellular basis for estrogenic regulation of neuronal growth and differentiation in higher cortical brain regions.

We have previously shown, utilizing *in vitro* binding assays, elevated ER levels in the neonatal rat hippocampus during the first postnatal week (O'Keefe and Handa, 1990). This finding provided biochemical evidence that the hippocampus has the capacity for estrogen responsiveness early in development. Specifically, ER concentration more than doubled between birth and postnatal day (PND) 4 and then declined precipitously between PND-7 and 10 to reach the low levels characteristic of the adult.

The elevation in hippocampal ER protein concentration during the early neonatal period could occur by several mechanisms. For example, ongoing neurogenesis in cells eventually differentiating into the ER phenotype during the first few days of life would further augment the total ER protein concentration. A recent study by Shughrue et al. (1990) demonstrating increased numbers of ER-containing neurons in cells apparently migrating from the cortical plate might support this possibility. Alternatively, ER gene transcription in individual neurons may be upregulated during the first postnatal week.

The subsequent reduction in hippocampal ER protein levels in the second postnatal week might also be due to several factors. If absolute ER levels remained unchanged,

an increase in neuronal proliferation and protein mass during development would result in an apparent reduction in ER content. This notion is possible given that neurogenesis in the dentate gyrus granular layer continues into adulthood (Bayer et al., 1982; Schlessinger et al., 1975). However, hippocampal ER numbers were also found to decrease during the second postnatal week when estradiol binding was expressed relative to hippocampal DNA content (O'Keefe and Handa, 1990), making this potential explanation unlikely. Another possible explanation is that there might be selective cell death in estradiol-concentrating neurons. Alternatively, the alterations in ER protein concentration may be secondary to concomitant changes in expression of the ER gene. None of these potential explanations are mutually exclusive and a combination of developmental mechanisms may be operating to cause the transient expression of ER protein in the hippocampus during the first postnatal week.

In the present study we have examined the ontogenetic profile of ER mRNA levels in the rat hippocampus in order to provide an index of ER gene expression in this region over the early postnatal period. Our hypothesis is that the developmental alterations in hippocampal ER protein content are a result of changes in ER gene transcription. This study was accomplished with a highly sensitive RNase protection assay which allowed us to absolutely quantify steady-state ER mRNA levels.

Materials and Methods

Animals

Sprague-Dawley (Sasco, Omaha, NE) adult males and neonatal male and female animals obtained from timed-pregnant dams were used in these experiments. Animals were maintained on a 12:12 hr light/dark cycle. Food and water were available ad libitum. On the day of birth pups were culled to approximately 8 per litter. Animals were killed on postnatal days (PND) 0, 4, 10 and in young adult males (approximately 2-3 months) by decapitation. Postnatal days were calculated using the day of birth as PND-0. Adult females were not used in this study to eliminate the possible effect of the changing hormonal milieu during the estrous cycle as a variable in hippocampal ER mRNA content. Indeed, estrogen has been shown to downregulate ER mRNA levels in the arcuate and ventromedial nuclei of the hypothalamus in adult female rats (Simerly and Young, 1991).

RNA Isolation

Total cellular RNA was isolated according to the guanidine isothiocyanate (GITC)/ cesium chloride method of Chirgwin, et al. (1979). Hippocampal tissues from 3-5 animals of the same sex (neonatal animals) or 2-4 (adult males) were pooled for each RNA sample used for ER mRNA determination. Animals were killed by decapitation and their brains immediately removed and placed ventral side up

on an ice-cold brass plate. The entire dorsal and ventral hippocampal formation including Ammon's horn, the dentate gyrus and the subiculum was dissected out and immediately homogenized in sterile dounce tissue grinders in a 4 M GITC (guanidium isothiocyanate) lysing buffer containing 5 mM sodium citrate, 0.1 M beta-mercaptoethanol, and 0.5% sarkosyl. After centrifugation through a 5.7 M CsCl pad at 100,000 x g for 12-16 hours at 15°C, the RNA pellet was suspended in DEPC water, phenol:chloroform:isoamyl alcohol (24:24:1) extracted and ethanol precipitated. The resulting pellet was washed with 70% ethanol and resuspended in DEPC water. An aliquot was taken to quantify total RNA content by uv spectrophotometry at 260 nm. The RNA was stored at -70 °C until assayed for ER mRNA by RNAse protection.

Preparation of radiolabelled antisense cRNA probe to rat ER mRNA and synthetic ER mRNA used as standards

The rat uterine ER cDNA construct pRcER6 (Koike et al., 1987) was kindly provided by Dr. Masami Muramatsu (The University of Tokyo; Tokyo, Japan). This clone contains 2090 nucleotides, including a long open reading frame encoding 600 amino acid residues, a 210 nucleotide 5' untranslated region, and a 74 nucleotide 3' untranslated region. A 171 base pair fragment generated by restriction digestion with PstI corresponding to nucleotides 1542-1713 in the steroid binding domain was subcloned into the pGEM-3Z

(Promega, Madison, WI) vector at the PstI site (See Figure 1). Antisense cRNA probe complementary to ER mRNA was transcribed *in vitro* using the SP6 RNA polymerase enzyme and EcoR1 linearized plasmid. Sense strand ER mRNA used for generating standard curves was synthesized using T7 RNA polymerase and Hind III linearized plasmid.

In Vitro Transcription

For *in vitro* transcription of antisense probes 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT), 0.5 *u*g EcoR1 linearized plasmid DNA, 0.5 mM each of ATP, CTP, GTP, 2.0 uM cold UTP, 50 uCi [alpha-^{32}P] labeled UTP (800 Ci/mmol; Amersham), 10 units placental RNAasin placental ribonuclease inhibitor, and 15-20 units SP6 RNA polymerase were added in a total incubation volume of 10 ul. The reagents, enzymes and protocol were obtained from Promega (Madison, WI). The incubation was carried out for 1 hour at 37C. One ul of RNAase-free pancreatic DNAase I (10 mg/ml;BRL) was then added to the incubates and thoroughly mixed. This reaction was allowed to incubate for 20 min. at 37C. The percent of radioactivity incorporated was determined by trichloroacetic acid precipitation. Total yeast t-RNA was added as carrier and RNA was purified by phenol:chloroform:isoamyl alcohol extraction (24:24:1). Unincorporated label was removed by spun column chromatography of the aqueous phase through

Sephadex G-50. Following ethanol precipitation, the resulting RNA pellet was redissolved in 25 ul of DEPC H_2O. At this time an aliquot of the RNA was taken and counted on a scintillation counter to assess specific activity of the labelled probe. Another aliquot was run on a 5% acrylamide 7 M urea gel to determine integrity and length of probe. Only full length (>90%) transcripts were used for the assays. For *in vitro* transcription of sense RNA utilized for generating standard curves, large scale reactions using trace amounts of alpha ^{32}P-UTP or CTP and 0.5 mM each of ATP, CTP, GTP and UTP were performed; these were essentially the same as described above except that t-RNA was not added as carrier. Sense RNA content was analyzed via uv spectrophotometry at 260 nm and serial dilutions were made.

Ribonuclease (RNAse) Protection Assay

The RNAse protection assay used was a modification from that of Melton (1984). Known quantities of sense RNA standard (2.5 to 0.1 pg) or 10-20 ug of sample total RNA in duplicate were dissolved in buffer containing 80% formamide, 40mM Pipes (pH 6.4), 0.4 M NaCl, and 1 mM EDTA. An excess of cRNA probe labelled with ^{32}UTP (1×10^5 cpm/sample tube) was dissolved in the same buffer and added to give a final volume of 30 ul. Following denaturation at 80 C for 10 min, the mixture was incubated for 16-20 h at 45°C. After hybridization, 300 ul of 0.3 M NaCl, 10 mM Tris-HCL (pH

7.5), 5 mM EDTA, RNAase-A and RNAase-T1 (final concentration 40 ug/ml and 2 ug/ml, respectively) was added and the solution incubated for 60 min. at 30 C. The digestion was terminated by the addition of proteinase-K and SDS at final concentrations of 125 ug/ml and 0.5%, respectively, for 15 min. at 37 C. The hybridized products were extracted with phenol:chloroform:isoamyl alcohol and then precipitated with ethanol. The protected hybrids were resuspended in 10 ulof a denaturing formamide load buffer [80% formamide, 10 mM EDTA (pH 8.0), 0.2% bromophenol blue, 0.2% xylene cyanol], denatured at 95 C for 5 minutes and electrophoresed on a 5% polyacrylamide-7 M urea gel. Gels were then fixed by two, 5 minute washes in 7% acetic acid and dried. Counts were analyzed and computer-imaged on a Betascope 6000 (Betagen Corp., Waltham, MA). Typical analysis time for Betascope data collection was 2 hours. A comparison of protected counts from sample lanes with those from the standard curve allows the determination of ER mRNA levels. The latter were expressed as femtomoles mRNA/mg total cellular RNA. Certain gels were also exposed to Amersham Hyperfilm-Bmax (Amersham) film for autoradiography.

The rat ER construct prepared by subcloning and the RNase protection assay are schematically presented in Fig. 1. When linearized with EcoR1 and transcribed with SP6 polymerase, a 245-base cRNA antisense transcript is expected. This includes 171 bases complementary to the open

reading frame of the rat ER mRNA and 64 bases complementary to vector DNA. Two hundred twenty two nucleotides of the antisense probe are expected to be protected by the sense ER RNA standard. One hundred seventy one nucleotides are protected by samples with detectable amounts of ER mRNA. Hence samples lanes with ER mRNA display smaller protected bands than standard curve lanes.

A typical assay standard curve of sense ER RNA is shown in Fig. 2a. Note the lack of hybridization (protected band) in the t-RNA control (lane 6), the protected bands of a larger size (222 bases expected) in the standard curve with 2.5, 1.0, 0.5, 0.25 and 0.1 pg [or 1.34 to 33.38 attomoles (amol)] of sense ER RNA (lanes 1 through 5) than in the samples (lanes 8 through 10) containing detectable amounts of ER mRNA. A plot of the protected counts in a standard curve analyzed with the Betascope are shown in Fig. 2b. The standard curves were linear from 1.0 to at least 150 attomoles (10^{-18} moles) sense RNA. Further assay validation is shown in Fig. 3. Note that absence of digestion with RNAase A and T1 demonstrates a full length probe (lanes 1 and 2) and smaller protected bands are present in samples containing ER mRNA (lanes 3 through 8) in the presence of RNAse enzymes.

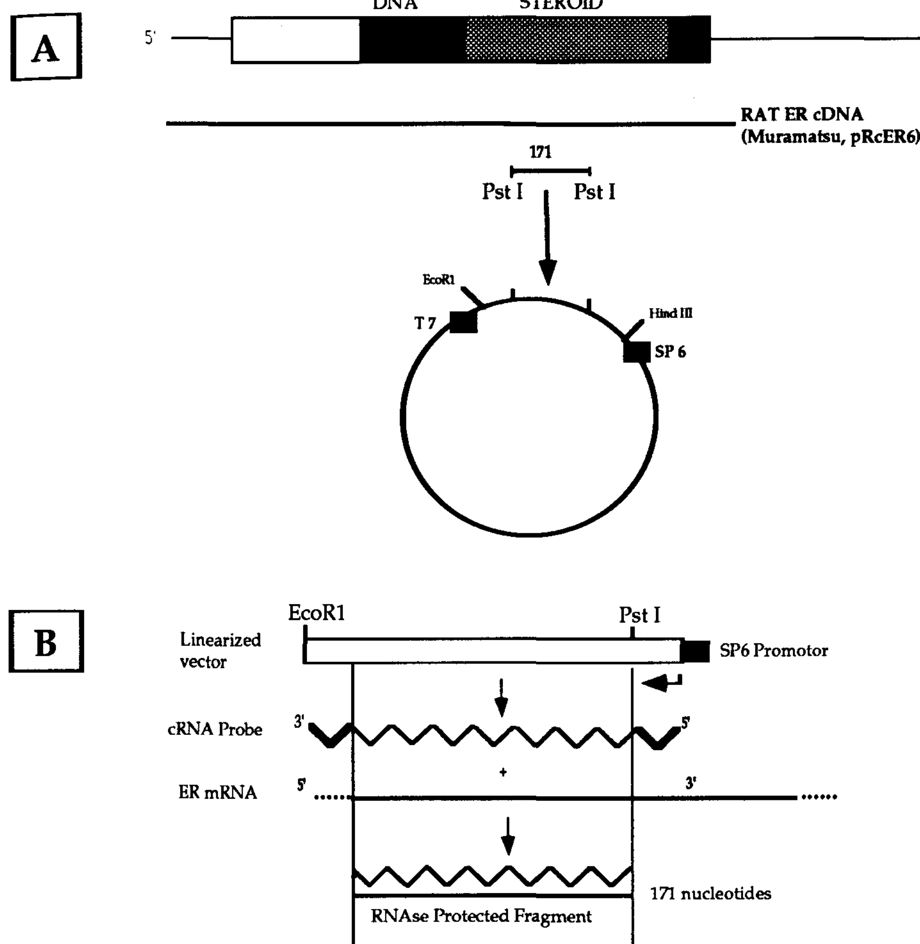
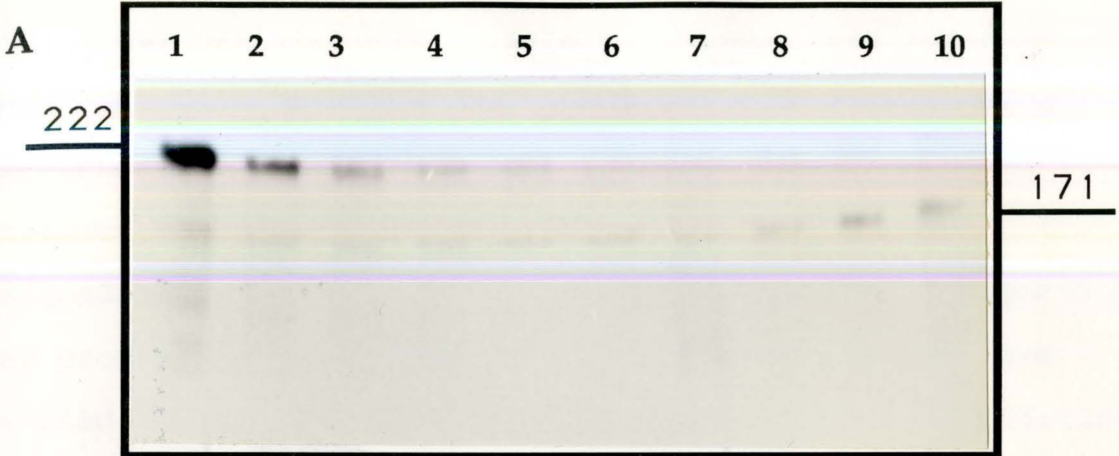


FIGURE 1. A. Schematic representation of the ER cDNA construct prepared by subcloning a 171 bp fragment of the rat uterine cDNA pRcER6, corresponding to nucleotides 1542-1713 in the coding region for the steroid binding domain. This fragment was generated by PstI restriction endonuclease digestion and subcloned into the corresponding PstI site of the pGEM-3Z vector.

B. RNAse protection assay: When linearized with EcoR1 and transcribed in vitro with SP6 RNA polymerase this construct is expected to produce a 245 base- long antisense cRNA transcript (171 complementary to ER mRNA and 64 to vector sequences). Hence, 171 bases of the probe will be protected by samples containing detectable ER mRNA.

A



B

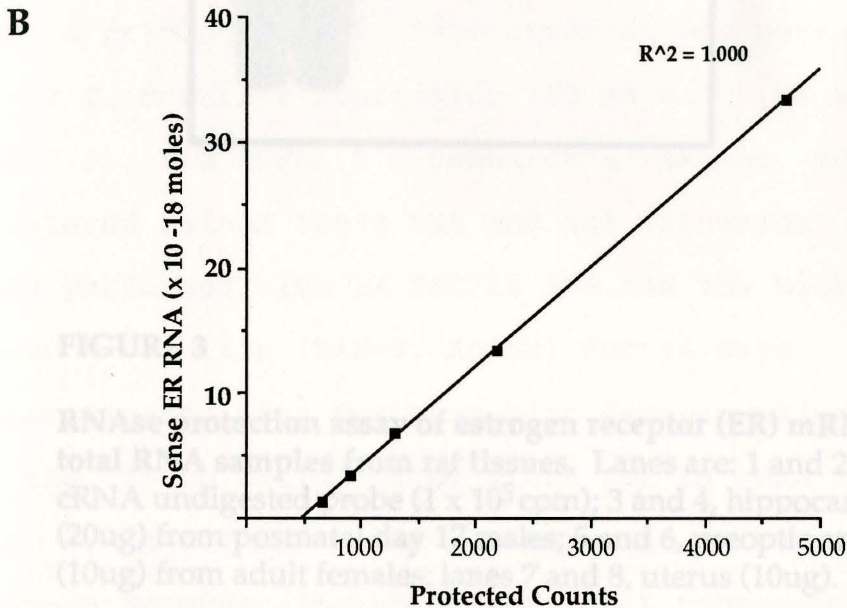


FIGURE 2

RNAse protection assay standard curve of synthetic sense rat estrogen receptor (ER). Samples of synthetic sense RNA were hybridized with excess radiolabeled antisense RNA probe and digested with RNAse, according to the procedures described in *Methods*. A. Autoradiogram of gel electrophoresis. Lanes 1 to 5 represent decreasing amounts of sense RNA added [2.5 to 0.1 pg or 33.38 to 1.335 attomoles (10 -18 moles)]. Lane 6 is the t-RNA control, lanes 7-10 are hippocampal samples.

B. The amount of radioactivity (protected counts) detected by Betascope analysis is plotted versus the quantity of sense RNA added to each hybridization.

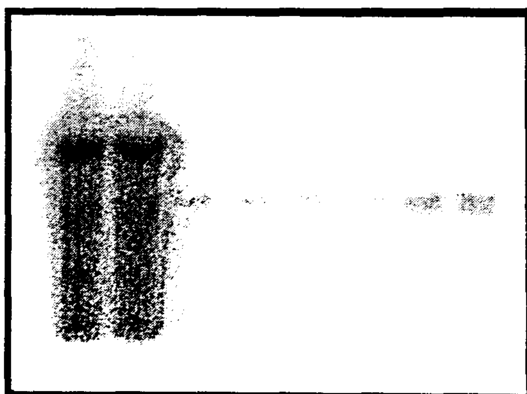


FIGURE 3

RNAse protection assay of estrogen receptor (ER) mRNA in total RNA samples from rat tissues. Lanes are: 1 and 2, the cRNA undigested probe (1 x 10^5 cpm); 3 and 4, hippocampus (20ug) from postnatal day 17 males; 5 and 6, preoptic area (10ug) from adult females; lanes 7 and 8, uterus (10ug).

Northern Blot Analysis

Total cellular RNA of selected tissues was isolated as described previously. RNAs (10-20 ug) were fractionated by electrophoresis through a 0.8% agarose-formaldehyde gel followed by capillary transfer to a nylon membrane (MagnaGraph, Micron Separations Inc.) with 10 x SSC (pH 7). The probe used for hybridization was the 171 bp fragment excised from the rat ER pcR6 cDNA construct with restriction endonuclease PstI, gel purified, electroeluted, and labeled with [^{32}P]-CTP to a specific activity of 1.0 x 10^8 cpm/ug by random priming (BRL). Hybridization was performed overnight at 42 C in buffer containing 750 mM NaCl, 50 mM Na phosphate (pH 7.0), 5mM EDTA, 5 x Denhardt's reagent, 50 ug/ml denatured salmon sperm DNA and 50% formamide. Final washes were performed with 1X SSC/1% SDS and the blot was placed under X-ray film (XAR-5, Kodak) for 14 days. An autoradiogram of a Northern blot demonstrating specificity of the 171 bp radiolabeled probe for ER mRNA is shown in Fig. 4. A 6.2 kB message is detected in samples that contain ER mRNA, including the adult hippocampus (lane 5). This size is in accordance with that described for the rat ER mRNA (Koike et al., 1987). In addition, the anterior pituitary was found to contain a smaller (approximately 4.7 Kb) form of the ER mRNA which is consistent with previous reports (Shupnik et al., 1989).

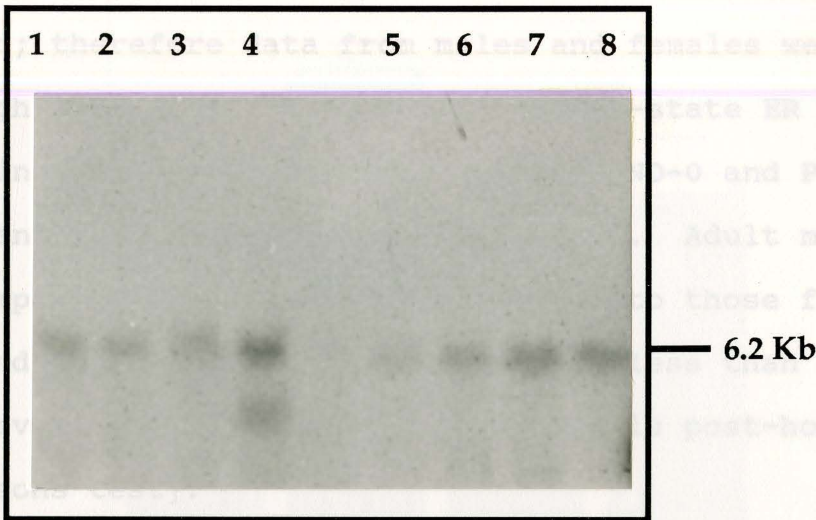


FIGURE 4

Northern Blot analysis of rat estrogen receptor (ER) mRNA. 20 ug samples of total RNA isolated from adult female rats were fractionated on an agarose/formaldehyde gel, blotted onto a nylon membrane and hybridized with a [32P]-labeled 171 bp ER cDNA probe as described in the text. Lanes 1 and 2, uterus; lanes 3, ovaries; lane 4, anterior pituitary, lane 5, hippocampus; lane 6, ovaries; lanes 7 and 8, uterus.

Results

The developmental profile of ER message expression in the hippocampus is shown in figure 5. No sex differences in ER message levels were found at the three postnatal ages examined; therefore data from males and females were pooled for graphic display. Hippocampal steady-state ER mRNA levels increased significantly between PND-0 and PND-4 ($p < 0.005$) and then declined again by PND-10. Adult male hippocampal ER mRNA values were similar to those found on PND-0 and PND-10 but were significantly less than ($p < 0.05$) PND-4 levels. [Two way ANOVA; Neuman-Keuls post-hoc comparisons test].

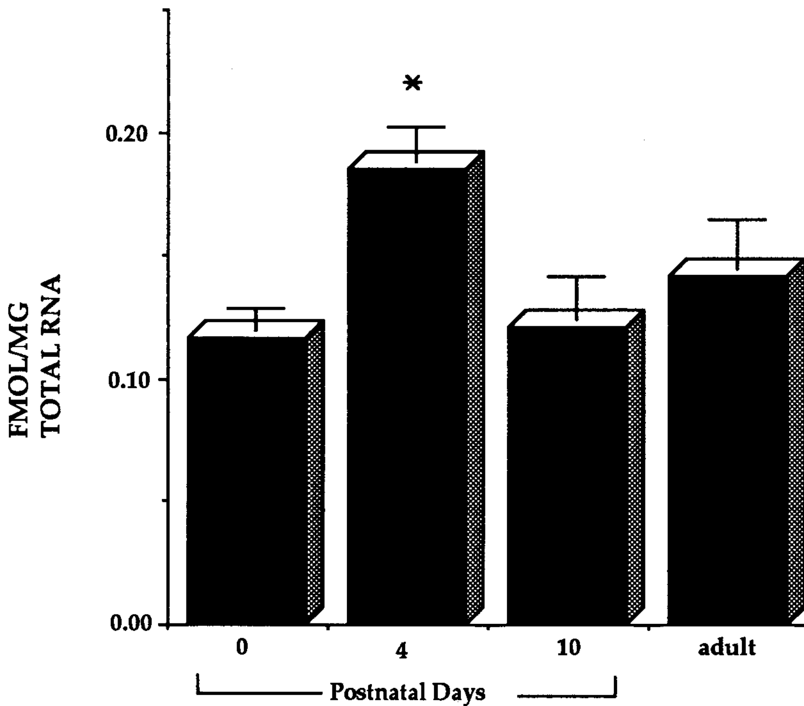


FIGURE 5

Steady-state estrogen receptor (ER) mRNA levels in the hippocampus of male and female postnatal and male adult rats. ER mRNA was quantitated by the RNAse protection assay described in the text. Each determination of ER mRNA content represents the mean of the duplicate samples; tissues from 2-5 animals of the same sex were pooled for each total RNA sample. No sex differences in ER mRNA levels were observed in neonatal rats; therefore, data from males and females were pooled. ER mRNA levels on postnatal day (PND)-4 (0.1848 + 0.014 fmole/mg total RNA; mean + S.E.M.; n= 5 males, 4 females) were significantly greater ($p < 0.005$) than those on PND-0 (0.1160 + 0.009; n = 4 males, 4 females) and PND-10 (0.1206 + 0.017; n= 5 males, 4 females), as well as in adult males (0.1412 + 0.0202; n= 8; $p < 0.05$) [Two way ANOVA; Neuman-Keuls post hoc comparisons test].

Discussion

This investigation demonstrates that hippocampal ER mRNA levels in the rat are transiently elevated on postnatal day 4. ER mRNA values increased from birth to PND-4 and then declined again by PND-10 to reach concentrations that were not significantly different from those found in the adult male. This temporal developmental pattern is similar to that previously found for hippocampal ER protein levels. (O'Keefe and Handa, 1990). Thus, we surmise that the ontogenetic changes previously reported for hippocampal ER protein concentration involve alterations in ER gene expression.

Lack of observable sex differences in hippocampal ER mRNA levels between PND-0, 4 or 10 are consistent with our previous results showing similar ER protein content in the male and female hippocampus throughout the early developmental period and in adult animals (O'Keefe and Handa, 1990). Adult females were not used in this study to eliminate cycling estrogen (or other reproductive hormone) levels as a variable in hippocampal ER mRNA content. The downregulation of ER mRNA levels by estrogen has been demonstrated in certain hypothalamic regions in adult female rats (Simerly and Young, 1991). Therefore, a sex difference in adult hippocampal ER mRNA levels is possible. In addition, we cannot rule out the possibility that subtle sex differences exist in individual neurons of the neonatal

brain which were beyond the detection sensitivity of the RNAase protection assay. Quantitative anatomical localization studies are necessary to determine if gender differences exist at the level of the individual cell.

Although the ontogenetic pattern for ER message levels found in this study and that previously reported for protein levels (O'Keefe and Handa, 1990) were similar, the magnitudes of the changes displayed some quantitative differences. ER protein levels increase approximately 2-fold between birth and PND-4. In this study message levels increased by approximately the same proportion (1.5 fold). However, protein levels between PND-4 and adult animals declined by 80% while the corresponding mRNA values observed here represent only a 25% reduction between these same ages. These apparent discrepancies imply that the magnitude of alterations in message expression does not precisely correlate with that of the encoded protein. Differences between message and protein levels have been noted for both the ER in a few brain regions including the hippocampus of the adult rat (Simerly, 1990; Pfaff and Keiner, 1973; Stumpf et al., 1974; Loy et al., 1988; Pelletier et al., 1988; Maggi et al., 1989).

This investigation, to our knowledge, is the first of its kind utilizing highly quantitative RNAase protection assays to measure ER in the brain. Recent studies by Simerly et al. (1990), using *in situ* hybridization, have

reported light to moderate labelling for ER mRNA in the ventral subiculum and presubiculum, the pyramidal layer of fields CA1, CA2 and CA3, and the polymorph layer of the dentate gyrus. In contrast, Loy et al.(1988), using steroid autoradiography to localize the ER protein in the young adult hippocampus, found labelled cells in CA1, but few in CA3 and none in CA2. Therefore, the amount of ER message might be greater than that of the protein in certain hippocampal regions of the adult. The difference between protein and mRNA levels suggest that additional regulatory mechanisms might be operating in concert with reductions in steady-state mRNA levels following PND-4 to depress total ER protein concentration. For example, ER gene transcription might be relatively high but posttranscriptional mechanisms such as reductions in message stability resulting in a shorter half life would further reduce the amount of message available for protein translation. Alternatively, posttranslational modifications such as a heightened receptor turnover rate might reduce ER protein levels.

The underlying mechanisms for the developmental alterations in hippocampal ER gene expression found in this study are not known. Variations in steady-state mRNA levels could be brought about by changes in synthesis or degradation of the message. This study has not examined whether regulation of the hippocampal ER gene is directly controlled at the level of gene transcription. However, a

plausible transcriptional developmental event might be the expression of trans-acting regulatory factors that suppress the ER gene following the first postnatal week or a positive regulatory molecule which is itself down-regulated during development. The specific regulatory factors which influence the ER gene in neural tissues are just beginning to be understood.

Gonadal steroids have been shown to regulate ER mRNA expression in the brain (Simerly and Young, 1991). This raises the possibility that the changing hormonal milieu during the perinatal period may contribute to the developmental changes in the hippocampal ER. However, if gonadal steroids were driving the developmental alterations in hippocampal ER message and thus protein levels, one might expect to see a sex difference in ER protein levels since plasma testosterone levels are higher in males than females from the 18th day of gestation through PND-5 (Weiscz and Ward, 1980; Dohler and Wuttke, 1975). We previously failed to detect a sex difference in hippocampal ER protein levels throughout the first two postnatal weeks (O'Keefe and Handa, 1990) suggesting that estrogen or testosterone are not inducing the transient rise (and subsequent decline) in ER protein levels. Moreover, gonadectomy on the day of birth does not alter the temporal pattern of ER ontogeny in the hippocampus (unpublished observations) and neural ER develop in hypothalamic transplants independently of the hormonal

state of the host animal (Paden et al., 1985). These observations further rule out a hormonal basis as the underlying mechanism for developmental alterations in ER expression.

It is feasible that other gene regulatory proteins such as those for the nerve growth factors and their receptors may regulate ER transcription given their overlapping distribution in the developing nervous system including the hippocampus (Miranda et al., 1991; Toran-Allerand, 1991). Indeed, the recent demonstration that nerve growth factor (NGF) upregulates ER mRNA levels in pheochromocytoma (PC) 12 cells (Sohrabji, et al., 1991) may support this possibility. In addition, concurrent NGF and estrogen administration to explant cultures of the embryonic septum and diagonal band was found to increase the number of cells expressing the ER message as well as enhance mRNA levels in individual neurons compared to either ligand alone (Toran-Allerand, 1991). This would suggest a synergistic role for estrogen and NGF in upregulating ER gene expression. Therefore, interactions with endogenous growth factors may play a role in the developmental regulation of the ER gene.

The reasons and mechanism(s) for the transiently elevated ER mRNA and protein levels followed by an apparent programmed loss of estrogen sensitivity in the hippocampus are unclear. Changes in ER gene transcription during

development might simply reflect the individual genetic program of neurons which express the ER phenotype. In accordance with this hypothesis, our recent work demonstrating intrinsic development of the ontogenetic profile in ER protein concentration in heterochronic hippocampal and neocortical transplants (O'Keefe et al., 1992, submitted) supports an inherent, genomic control in the regulation of ER levels during development. Estrogen appears to be important for neuronal differentiation, process formation and synaptogenesis (Faivre et al., 1981; Toran-Allerand, 1976,1983, Uchibori and Kawashima, 1985; Reisert et al., 1987; Blanco et al., 1990; Ferreira and Caceras, 1991) in hormone sensitive target cells. Once these developmental events have been "programmed" or are complete, a subset of estrogen target neurons might cease expression of the ER.

Another possible explanation for the suppression of hippocampal ER mRNA and protein concentration following the first postnatal week is that there might be selective cell death in estradiol-concentrating neurons. Although this event would appear to contradict the current dogma that gonadal steroids function in promoting neuronal survival (Toran-Allerand, 1984; Gorski, 1985; Hauser and Toran-Allerand, 1989), insufficient levels of circulating estrogen or testosterone during this period might render the ER-containing subset of hippocampal neurons more susceptible to

cell death.

Further studies on the putative factors participating in the ontogenetic regulation of the ER gene will provide crucial insights into the underlying molecular mechanisms for site-specific sexual differentiation of the CNS.

CHAPTER V

THE ONTOGENY OF ESTROGEN RECEPTORS IN HETEROCHRONIC HIPPOCAMPAL AND NEOCORTICAL TRANSPLANTS DEMONSTRATES AN INTRINSIC DEVELOPMENTAL PROGRAM

Abstract

We investigated the intrinsic versus environmental regulation of estrogen receptor (ER) ontogeny in the neocortex, hippocampus and hypothalamus by employing a heterochronic transplantation paradigm. These studies were based on previous reports demonstrating that neural ER develop asynchronously with quantitatively distinct ontogenetic profiles in various brain regions. Fetal (E14-15) hippocampal, frontal cortical or hypothalamic-preoptic area (HPOA) primordial tissue was grafted into frontal cortical lesion cavities made in newborn (PND-0) rats. Thus, the grafted tissue was 1 week younger than the host. Two and 4 weeks following transplantation surgery which corresponds to a theoretical donor age of PND-7 and PND-21, the grafts, a region of the host neocortex surrounding the transplant, and the host hippocampus, frontal cortex or HPOA (depending on graft type), were assayed for ER content using *in vitro* binding assays. ER concentration in hippocampal grafts at theoretical age PND-7 (4.36 ± 0.68 fmol/mg protein) were significantly higher ($p < 0.01$) than those

found in the host (PND-14) hippocampus (1.66 $\pm$ 0.2) and in the host neocortex adjacent to the transplant (1.34 $\pm$ 0.27). By theoretical graft age PND-21, ER concentration in hippocampal transplants had decreased to levels (1.24 $\pm$ 0.18) comparable to those found in the host. This developmental pattern is analogous to that previously reported for the *in situ* hippocampus. A similar profile of ER concentration corresponding to the donor age developmental timetable was observed in neocortical grafts. ER levels in HPOA grafts did not change from theoretical donor age PND-7 to PND-21 which also corresponds to the normal ontogenetic profile. These data suggest that region-specific developmental patterns of ER expression in the brain are specified very early in embryonic development.

Introduction

A major quest in neurobiology is to dissect the influence of a neuron's genetic program from that of the environment on its developmental outcome. For example, by examining parcellation of the cerebral cortex into distinct cytoarchitectonic, chemoarchitectonic and functional regions, several theories implicating both genetic and environmental factors have been proposed (Rakic, 1988; O'Leary, 1989; McConnell, 1990). Evidence provided by heterochronic transplantation studies (Chang et al., 1986; O'Leary and Stanfield, 1989; Schlagger and O'Leary, 1991) suggests that epigenetic factors operate upon an

equipotential "protocortex" during development to regulate the differentiation into unique cortical areas seen in the adult (O'Leary, 1989). The requirement of sensory afferent input and an intact peripheral receptor apparatus for the formation of distinct neocortical cytoarchitectural features such as somatosensory cortical barrels in the rodent supports this hypothesis (Van der Loos and Welker, 1985; Killackey, 1985), although recent studies of the primate visual cortex (Kuljis and Rakic) suggests that this may not be universal across cortical areas or species.

Cerebral cortical neurons become restricted to both a distinct cytoarchitectonic based molecular phenotype (Barbe and Levitt, 1991) and to a particular laminar fate (Chang et al., 1986; McConnell, 1985, 1988, 1990) very early in embryonic development. This suggests intrinsically regulated mechanisms for these developmental features which may be dependent on stage of the cell cycle (McConnell and Kazsnowski, 1991). Local environmental cues, however, have been shown to influence ultimate neurotransmitter phenotype and cytodifferentiation of immature neuroblasts in the peripheral nervous system (DiCiccio-Bloom et al., 1990). In addition, neurotransmitter phenotypic interconversion in response to epigenetic factors has been demonstrated in developing cerebral cortical neurons (Iacovitti et al., 1989). Recent work (McConnell and Kaznowski, 1991) indicates that there is a narrow window of time during which

the environment specifies information required for the layered patterning of the cerebral cortex. It remains to be clearly established whether neuronal progenitors in the cerebral cortex or diencephalon require instructive input from the local milieu for ultimate molecular phenotype selection.

The developmental decision for a neuron to express the estrogen receptor (ER) phenotype has not been previously examined. The ER system plays a paramount role in establishing sex specific differences in the brain (Toran-Allerand, 1984; MacLusky and Naftolin, 1981). Given the importance of the ER in this developmental process, we are attempting to understand the mechanism(s) by which the receptor is regulated during ontogeny.

Differences in regional ER concentration in the brain suggest that neural estrogen sensitivity develops asynchronously. For example, the rodent hippocampus and neocortex display a transitory increase in ER levels during the first postnatal week (O'Keefe and Handa, 1990; MacLusky et al., 1979). The timing of elevated cortical ER levels coincides with a period of significant growth, synaptogenesis and differentiation in the neocortex and hippocampus (Blue and Parnevales, 1983; Miller, 1988). This temporal association suggests that estrogen may function as a trophic factor for cortical target cells during the early postnatal period. This concept has obtained support by *in*

vitro studies, which demonstrated enhanced neurite outgrowth in cerebral cortical cultures in response to estrogen (Toran-Allerand, 1984; Uchibori and Kawashima, 1985; Blanco et al., 1990). In addition, the presence of elevated receptor levels during the "critical period" for sexual differentiation supports a role for the ER in establishing previously reported gender differences in cognitive functions such as learning and memory (Beatty, 1984).

In contrast to the transient developmental pattern found in the hippocampus and neocortex, the hypothalamus, preoptic area and amygdala demonstrate pre- and postnatal increases in ER concentration which remain elevated into adult life (MacLusky et al., 1979; Vito and Fox, 1982; Friedman et al., 1983). The ER in these areas mediates the developmental neural organization for sex specific patterns of gonadotropin secretion and reproductive behaviors (Toran-Allerand, 1984; MacLusky and Naftolin, 1981). However, the factors governing the developmental appearance of estradiol concentrating neurons and the site dependent expression of ER are largely unknown.

Intracerebral grafting is a powerful tool to address inquiries regarding normal developmental mechanisms within the CNS (Gage and Fisher, 1991). The technique has been particularly advantageous to investigate parcellation of the cerebral cortex (Schlagger and O'Leary, 1991; Castro et al., 1991), specificity of neuronal connections (Chang et al.,

1986; O'Leary and Stanfield, 1989), commitment of neurons to a distinct phenotypic fate (McConnell, 1985, 1988; McConnell and Kaznowski, 1991; Barbe and Levitt, 1991), neurotransmitter differentiation (Hohmann, 1989) and senescent alterations in brain function (Granholm et al., 1987). In the present study we have exploited the divergent patterns of ER phenotypic expression in the cerebral cortex versus the hypothalamus to examine the commitment of neural tissue to a specific timetable for ER ontogeny. Embryonic day (E) 14-15 primordial tissues from the presumptive hippocampus, frontal cortex, or HPOA were transplanted into frontal cortical sites in newborn (PND-0) hosts allowing us to address the question of whether neurons are preprogrammed for a specific developmental expression of the ER phenotype or whether extrinsic factors modify the timing of receptor ontogeny.

Materials and Methods

Animals

A total of 30 pregnant dams and 225 newborn host rats were used in these studies. Timed pregnant Sprague-Dawley rats (Sasco, Omaha, NE) were housed individually in a 12:12 hr light:dark cycle with free access to food and water. Embryonic day (E) 0 is defined as the day the mothers were found to be sperm positive. The day of birth is considered to be postnatal day (PND) 0.

All host animals, except those used for histological

evaluation, were bilaterally gonadectomized under ether anesthesia 20 to 24 hr prior to sacrifice. Gonadectomy was performed to remove endogenous gonadal steroids, which could interfere with the *in vitro* binding assay used for ER quantification. It also serves to recycle any DNA bound receptor to the unoccupied form.

Transplant paradigm

Fetal (E14-E15) neuronal grafts were placed into right frontal cortical sites in newborn (PND-0) hosts. Homotopic (frontal cortical) and heterotopic [hippocampal and hypothalamus-preoptic area (HPOA)] transplants were used. In the normal animal the hippocampus and neocortex have an analogous developmental expression of ER while the HPOA demonstrates a divergent pattern of ER ontogeny (MacLusky et al., 1979; Vito and Fox, 1982; O'Keefe and Handa, 1990). The selection of these tissue types for transplantation allowed us to examine ER levels in: (i) heterotopic cortical grafts (hippocampal transplants), (ii) homotopic cortical grafts (frontal cortical transplants), and (iii) heterotopic non-cortical grafts (HPOA transplants).

ER levels in transplants were analyzed at 2 and 4 weeks post-grafting which corresponds to a theoretical tissue age of PND-7 and PND-21, respectively. These dates were chosen as ER numbers are normally high in all 3 brain regions on

PND-7, remain high in the PND-21 HPOA, but decline to extremely low levels in the neocortex and hippocampus by PND-21 (MacLusky et al., 1979; Vito and Fox, 1982; O'Keefe and Handa, 1990). In addition, ER content was determined in the same graft-type region of the host brain as well as in the neocortical region surrounding the transplant. This was done for purposes of comparison and to examine whether the transplant could potentially induce alterations in ER levels in the adjacent areas of the host brain.

Transplant procedure

Donor tissues from embryonic day 14-15 (crown rump length of 13-15 mm) fetuses were used for grafting. The sex of the donor animals was not determined. Pregnant rats were anesthetized with sodium phenobarbital (50 mg/kg body weight) and the fetuses were individually removed and placed in 25^{0}C sterile Ringer's solution. The fetal skull was opened with microsurgical instruments under a surgical microscope and the meninges were removed. Blocks of hippocampal, frontal cortical, or HPOA primordial tissue were dissected and aspirated into a glass cannula fitted onto a Hamilton microsyringe. Male and female neonatal graft recipients were anesthetized under hypothermia. A midsagittal scalp incision was made and a bone flap opened with a scalpel. Small right frontal cortical (1mm rostral to bregma and 1.5 mm lateral to the sagittal suture)

aspiration lesions were made with a glass pipet. The transplants were slowly injected into the lesion cavity with the syringe attached to a micromanipulator. The grafts were held in place with the bone flap and the incision was sutured with 4-0 silk suture. The graft recipients were returned to their mothers following the surgical procedure. This transplant procedure has previously been described previously (Castro et al., 1991).

Tissue dissection

Animals were killed by decapitation at 2 and 4 weeks post-grafting. The brains were immediately removed and placed on an ice cold brass plate with the brain placed dorsal side up. With the aid of a surgical microscope the transplants were located and a coronal brain slice was made just anterior and posterior to the transplant. The transplant was removed as a wedge of tissue with care taken not to include the surrounding region of the host neocortex. An adjacent region of the host neocortex was removed using a 1 mm stainless steel punch. The host hippocampus, opposite homotopic frontal cortex, and HPOA were dissected in the appropriate groups of animals. The entire dorsal and ventral hippocampus was dissected and included the subicular complex, Ammon's horn, and the dentate gyrus. The opposite homotopic neocortex in same location as the transplant site was dissected with a 1 mm punch. The HPOA consisted of the

preoptic area (POA) and medial basal hypothalamus (MBH). The POA was removed as the region just anterior and posterior to the optic chiasm using the anterior commissure as its dorsal limit and extending laterally to 1 mm on either side of the third ventricle. The MBH was dissected as a wedge of tissue just posterior to the optic chiasm and anterior to the mammillary bodies with the top of the third ventricle as the dorsal limit and the hypothalamic sulci as lateral boundaries.

Cytosolic estrogen receptor (ER) binding assay

Tissues from 1-3 animals of the same sex (depending on transplant size) were used for each determination of ER content. ER levels were quantified using a modification of procedures described by MacLusky and McEwen (1979) which has been previously reported (O'Keefe and Handa, 1990).

Saturation Analysis

Saturation analysis of [^{3}H]-estradiol binding was determined in hippocampal and neocortical transplants and corresponding regions of the host brain at the 2 week survival time (host postnatal age PND-14). Tissues from 8-12 animals of the same sex were pooled for the analysis. Cytosols were prepared and incubated with increasing amounts of [^{3}H]-estradiol (0.1 to 5.0 nM). Nonspecific binding was determined using 1 uM radioinert R2858, a specific ER

ligand. Data were analyzed with a computerized, statistically weighted regression analysis program, LIGAND (Munson and Rodbard, 1980).

Histology

Transplant histology was examined in a separate group of four week old animals that had received one of the 3 types of grafts at birth. Animals were anesthetized with sodium pentobarbital and perfused transcardially with a 0.9% heparinized saline solution followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4). The brains were removed and placed in a 30% sucrose solution for cryoprotection until they sank (approximately 24 hr) and stored at -70° C until sectioned. Forty micron thick coronal sections were made on a cryostat, mounted onto gelatin coated slides and stained with Cresyl violet.

Statistics

Differences in ER levels in each of the regions assayed were analyzed with a multifactor analysis of variance (ANOVA) with sex, age and tissue region as the 3 factors.

Results

Hippocampal transplants placed heterotopically in the neocortex

Estrogen receptor (ER) levels in hippocampal transplants at two weeks post-grafting, which corresponded

to a theoretical graft age of PND-7, were significantly greater (4.36 ± 0.68 fmol/mg protein) than those found in the host hippocampus (1.66 ± 0.2) and the neocortical region surrounding the grafts (1.34 ± 0.27)[Fig. 1]. ER levels in the hippocampal transplants at theoretical graft age PND-21 (4 weeks post transplantation) decreased to values (1.24 ± 0.18 fmol/mg protein) that were not different from the host hippocampus or neocortex. In these grafts as well as the neocortical and hypothalamic grafts, no sex differences in receptor concentration were found (multifactor ANOVA); therefore, data from males and females were pooled for illustrative purposes.

Neocortical transplants placed homotopically in the neocortex

A developmental profile of ER expression which was similar to that described above for hippocampal grafts was observed in the neocortical transplants (Fig. 2). At theoretical graft age PND-7, ER values were at least 5-fold higher (8.84 ± 2.8 fmol/mg protein; $p < 0.01$) in the grafts than in the opposite homotypic cortex (1.63 ± 0.30 fmol/mg protein) or in the neocortical tissue adjacent to the transplants (0.84 ± 0.20 fmol/mg protein). By 4 weeks post-grafting (theoretical age PND-21), ER levels in the frontal cortical transplants had significantly decreased (1.51 ± 0.23 fmol/mg protein; $p < 0.01$), but they were still higher than those in the opposite (0.64 ± 0.17; $p < 0.05$) and

adjacent (0.54 $\pm$ 0.14 ; $p< 0.01$) host neocortex.

HPOA Transplants placed heterotopically in the neocortex

The temporal pattern of ER expression observed in the HPOA transplants differed from the patterns found in hippocampal and neocortical grafts (Fig. 3). ER levels in these grafts were significantly lower ($p < 0.01$) than values found in the host HPOA at both two (3.81 $\pm$ 0.76 fmol/mg protein) and four weeks (3.23 $\pm$ 0.85) post-transplantation. Importantly, there was no difference in ER concentration between the theoretical graft ages PND-7 and PND-21.

When ER levels are expressed as fmol/mg DNA, a similar profile of ER development was observed in the hippocampal, neocortical and HPOA grafts (data not shown).

Affinity characteristics of ER in Transplants

Saturation analysis of [^{3}H]-estradiol binding in the hippocampal and frontal cortical grafts (Fig. 4) as well as in the corresponding region of the host brain (data not shown) at two weeks post-transplantation revealed an apparent dissociation constant [K_d] of $2.9 \pm 0.69 \times 10^{-10}$ M (n= 3) for the hippocampal grafts, 6.55×10^{-10} M (n=2) for the neocortical grafts, $4.77 \pm 2.32 \times 10^{-10}$ M (n=3) for the host hippocampus and 3.93×10^{-10} M (n=1) for the host neocortex.

Transplant histology

Hippocampal grafts showed a resemblance to a normal cytoarchitectural arrangement with apparent divisions into pyramidal and granule cell layers (Fig. 5A). Neocortical grafts contained characteristic whorls and layers of cells (5B) while HPOA grafts contained separation into groups of small and large neurons in what appeared to be nuclear subdivisions (5C). The transplants typically filled the lesion cavity and appeared well integrated with the host brain. In general, hippocampal and frontal cortical transplants were larger than HPOA transplants.

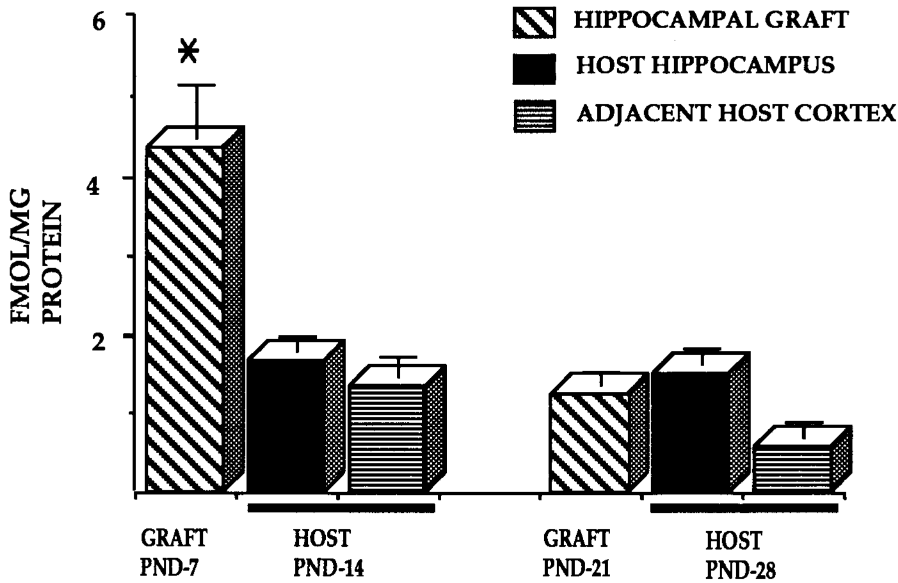


Figure 1

Cytosolic [3H]-estradiol binding (mean ± S.E.M.; fmol/mg protein; n=13-14/group) in hippocampal transplants, the host hippocampus, and the host neocortex adjacent to the graft at 2 (theoretical graft age PND-7, host age PND-14) and 4 (theoretical graft age PND-21, host age PND-28) post-transplantation. Tissues from 1-3 (depending on graft size) animals of the same sex were pooled for each determination. No significant sex differences were found in any of the groups; therefore data from males and females were combined for illustrative purposes. * $p < 0.01$ compared to all other groups (Multifactor ANOVA/ Tukey-Kramer post hoc comparisons).

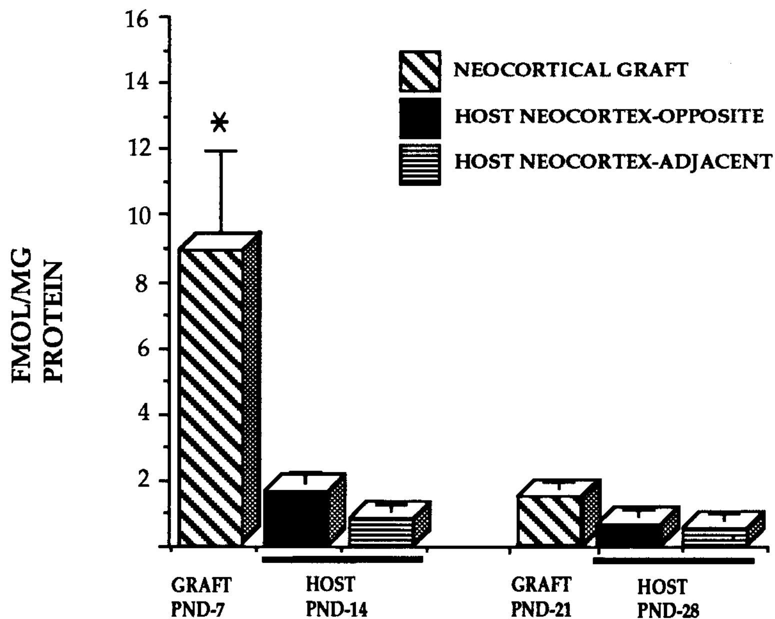


Figure 2

Cytosolic [3H]-estradiol binding (mean ± S.E.M.; fmol/ mg protein; n=14/group at 2 wks; n=11-12/group at 4 wks) in neocortical transplants, the opposite homotypic host neocortex and the host neocortex adjacent to the graft at 2 (theoretical graft age PND-7, host age PND-14) and 4 (theoretical graft age PND-21, host age PND-28) wks post-transplantation. No significant sex differences were found in any of the groups; therefore data from males and females were combined for illustrative purposes.
* $p < 0.01$ compared to all other groups (Multifactor ANOVA/Tukey-Kramer post hoc comparisons).

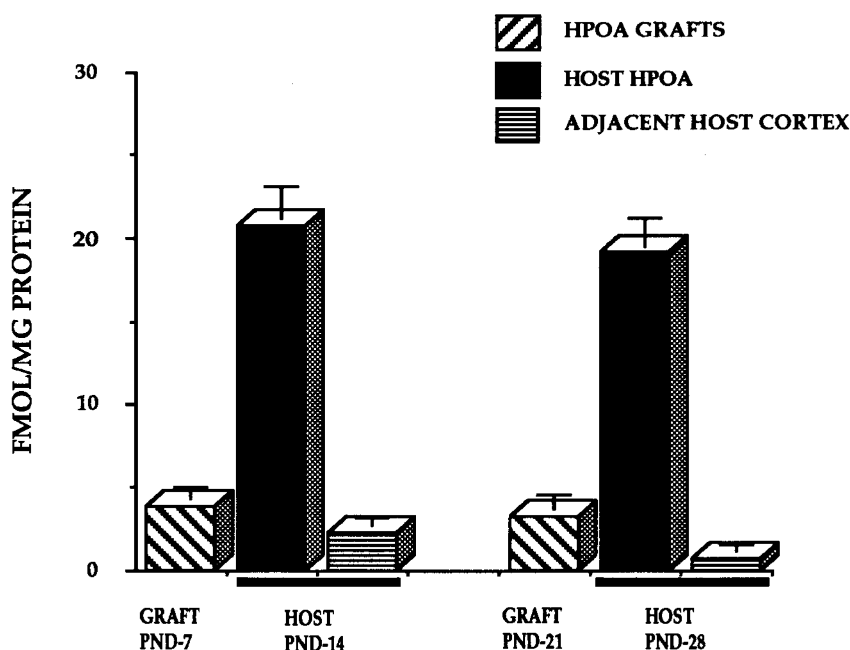


Figure 3

Cytosolic [3H]-estradiol binding (mean ± S.E.M.; fmol/mg protein; n= 11-13/group at 2 wks; n= 7/group at 4 wks) in HPOA transplants, the host HPOA, and the host neocortex adjacent to the graft at 2 (theoretical graft age PND-7, host age PND-14) and 4 (theoretical graft age PND-21, host age PND-28) wks post-transplantation. No significant sex differences were found in any of the groups; therefore data from males and females were combined for purposes of illustration. ER levels in the endogenous host HPOA were significantly greater than those in the grafts at both 2 and 4 wks post-transplantation ($p < 0.01$). Additionally, no difference in ER numbers between graft age PND-7 and PND-21 was found.

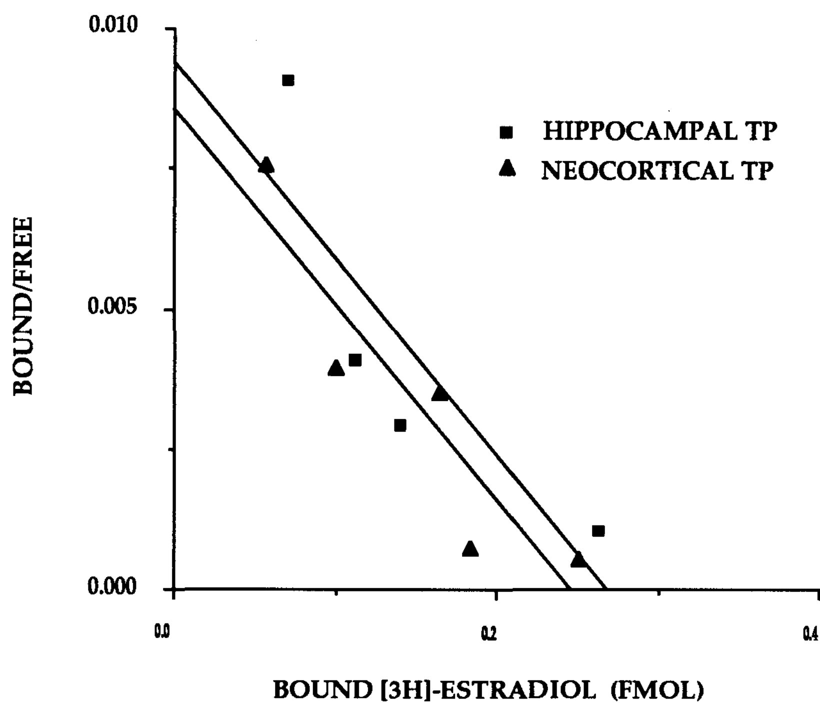


Figure 4

Representative Scatchard plots for specific [3H]-estradiol binding in cytosolic extracts from theoretical age PND-7/8 hippocampal (square) and neocortical transplants (triangle). Kd values were 1.88 x 10 -10 M and 1.91 x 10-10M, respectively. Tissues from 8-11 animals of the same sex were pooled for the analysis.

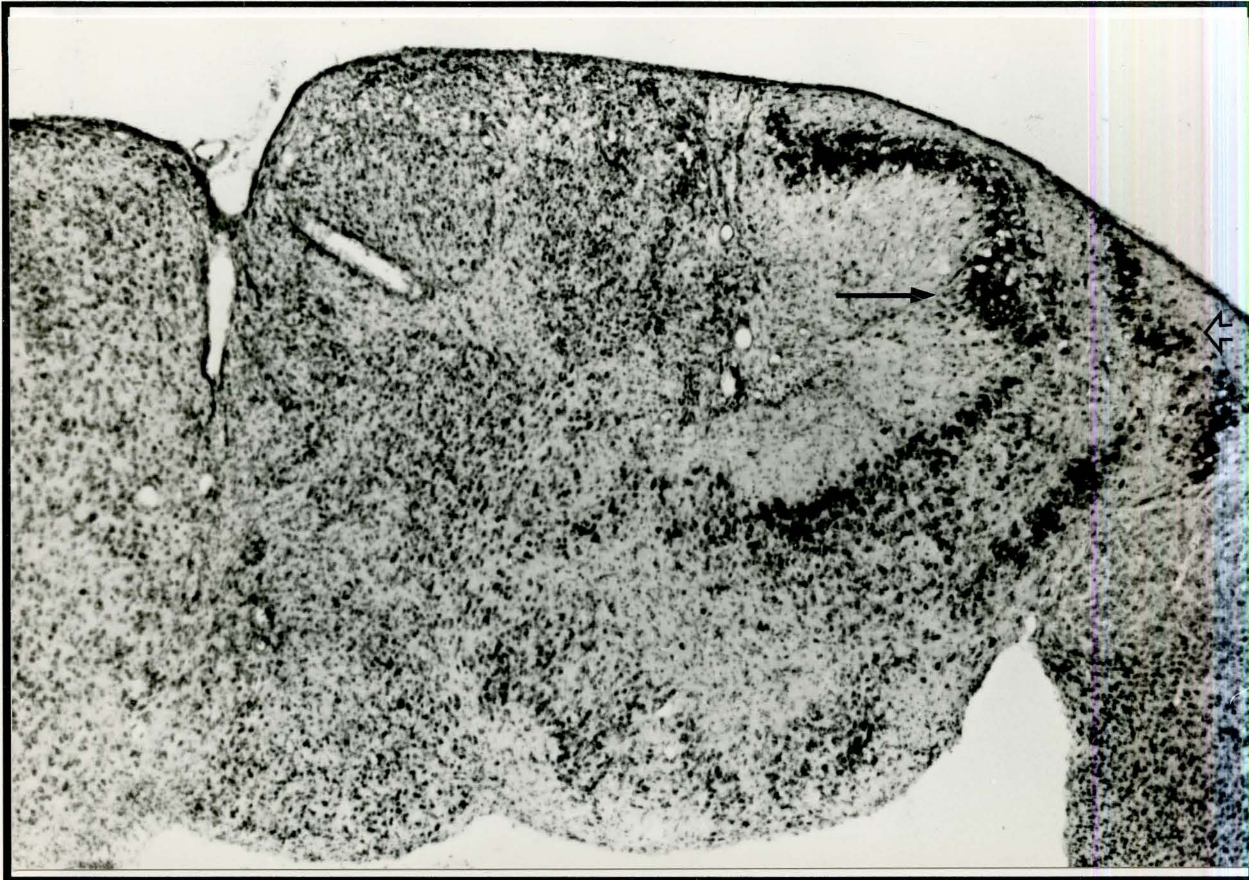


FIGURE 5A

Nissl stained section through a representative hippocampal graft placed into a right frontal cortical lesion site at 4 weeks post-grafting (theoretical age PND-21). Note the partial retainment of typical cytoarchitectural features in the hippocampal graft such as delineation into what appears to be a pyramidal cell layer (arrowhead) and densely packed granule cells (open arrow).

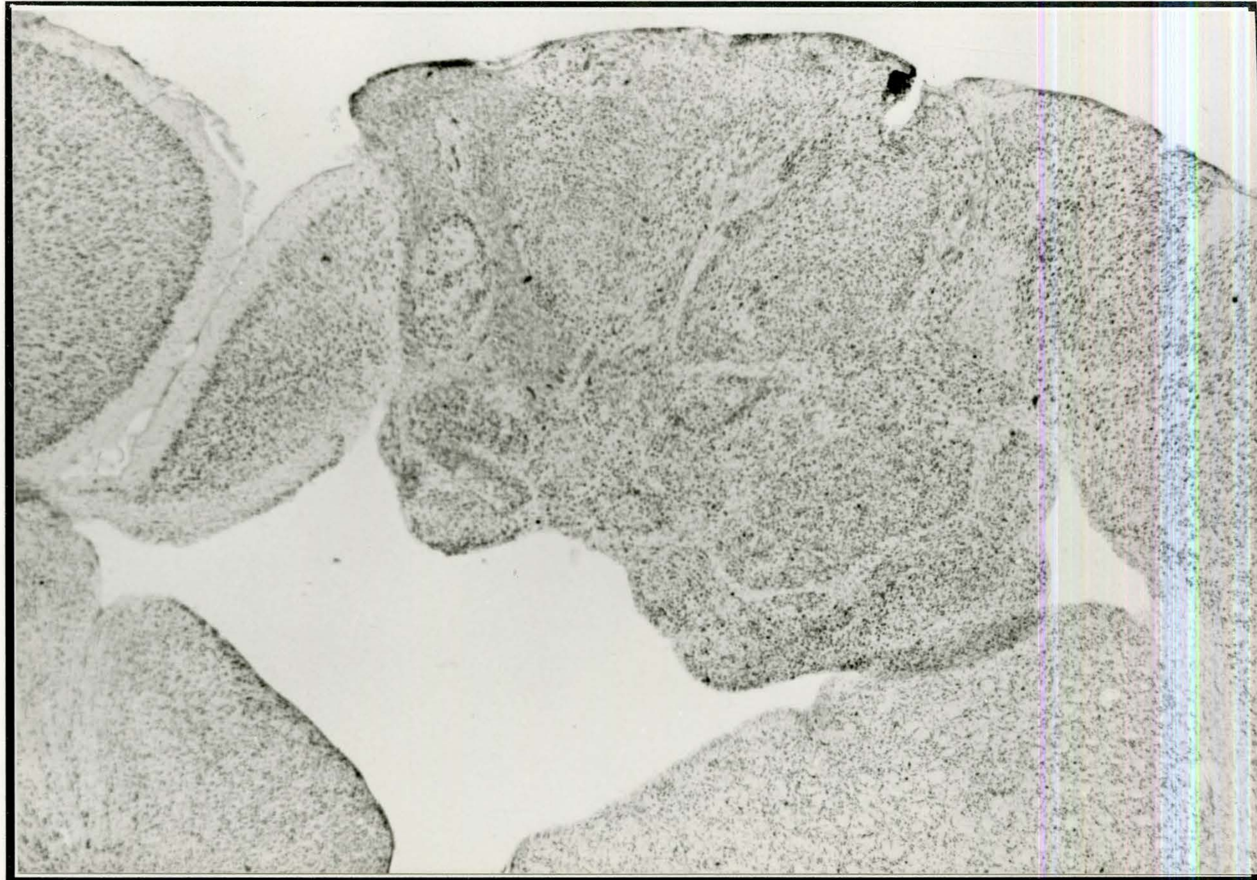


FIGURE 5B

Nissl stained section through a Neocortical Transplant placed into a right frontal cortical lesion site at 4 weeks postgrafting (theoretical age PND-21). Note the characteristic whorls and layers of cells in the graft.

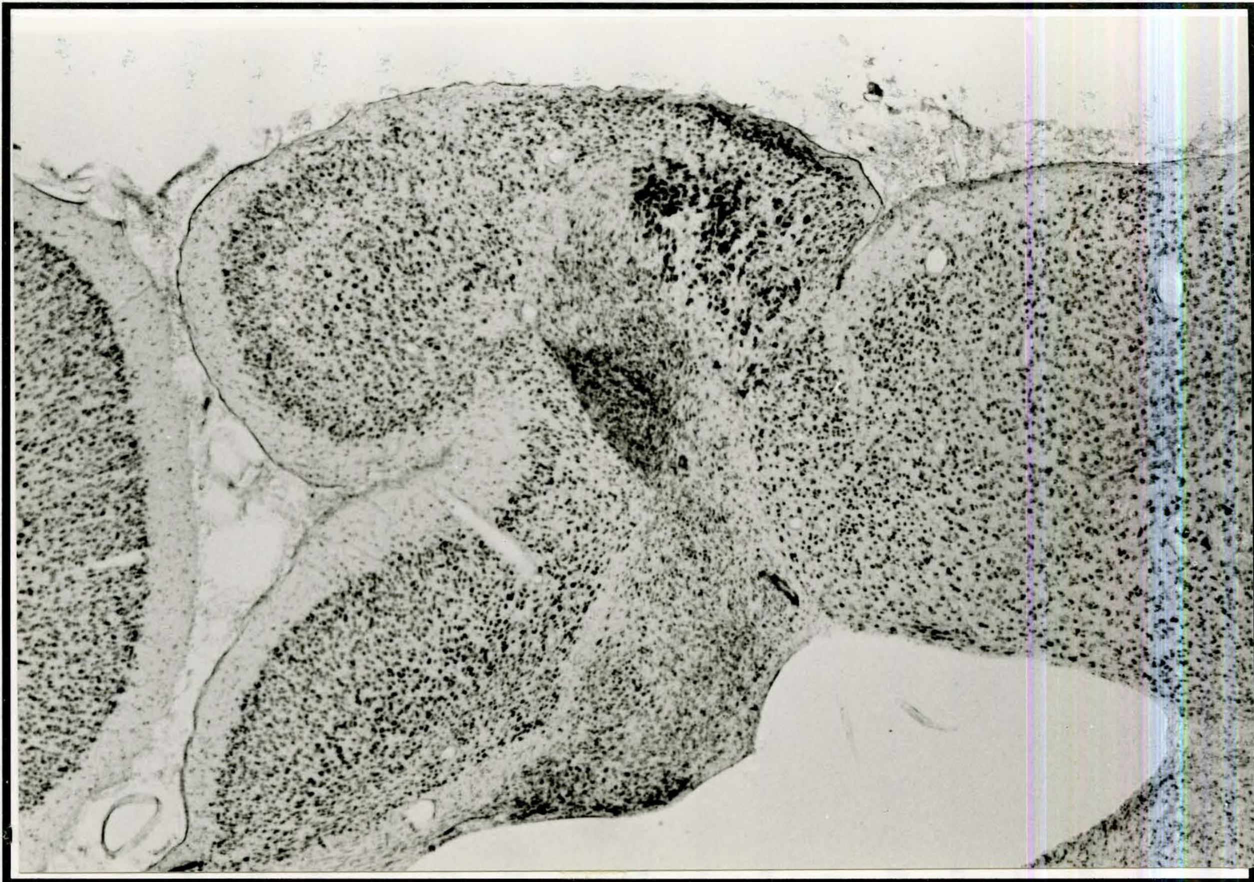


FIGURE 5C

Nissl stained section of a hypothalamic/preoptic area transplant grafted into a right frontal cortical lesion cavity at 4 weeks post-grafting (theoretical age PND-21). Note the division into small and large cell nuclear groups characteristic of the *in situ* hypothalamus and preoptic area.

Discussion

This study indicates that the timing of hippocampal and neocortical estrogen receptor (ER) development is intrinsically regulated. ER content in hippocampal and neocortical transplants was elevated at theoretical donor age PND-7 and declined to very low levels in the PND-21 grafts. This developmental pattern recapitulates the phenomenon found in the *in situ* rodent hippocampus and neocortex (MacLusky et al., 1979; O'Keefe and Handa, 1990). Since the donor age timetable was followed, the signal for a neuron to express the ER phenotype in a unique age-related fashion may be preprogrammed or specified very early in embryonic development.

Saturation analysis of $[^3H]$-estradiol binding in the hippocampal and frontal cortical transplants at 2 weeks post-transplantation demonstrates that the ER in the grafts represents a single, high affinity binding site for estradiol with the same binding characteristics as previously reported in normal CNS tissue (MacLusky et al., 1979; Friedman et al., 1983; O'Keefe and Handa, 1990). Thus, alterations in receptor affinity do not account for the change in ER concentration seen in the transplants between theoretical age PND-7 and PND-21.

In HPOA transplants there was no difference in ER numbers between theoretical age PND-7 and PND-21 grafts. These results suggest that the maximum potential for ER

expression had been reached at two weeks post-transplantation, the earliest timepoint evaluated. In related work, ER concentrations in the rat medial basal hypothalamus and POA increased steadily throughout the latter part of gestation and during the early postnatal period (MacLusky et al., 1979; Vito and Fox, 1982). Furthermore, most investigations have demonstrated that adult levels of ER were attained in the MBH by PND-4 to PND-7. One conclusion from our HPOA transplant data that is consistent with these past studies is that ER development in hypothalamic grafts is typical of the *in situ* HPOA where ER levels have peaked by PND-7. Alternatively, we may have selectively grafted tissue which contained a low percentage of estradiol concentrating primordial cells. This possibility appears unlikely given that the entire ventral and inferior germinal neuroepithelium surrounding the third ventricle was included in the grafts. Thus, stem cells, as well as some post-mitotic neurons, destined for the future periventricular nucleus, medial preoptic nucleus, paraventricular nucleus (PVN), sexually dimorphic nucleus of the POA, the ventromedial nucleus and the arcuate nucleus (Altman and Bayer, 1978 a,b), all of which contain a relatively high density of estrogen concentrating neurons (Pfaff and Keiner, 1973), should have been contained within the transplants.

The donor age chosen for transplantation in the present

study corresponds to the period prior to the onset of ER appearance in the neocortex (MacLusky et al., 1979) and before or near the earliest timepoint that estradiol concentrating neurons have been detected in the rat HPOA (MacLusky et al., 1979; Vito and Fox, 1982). The existence of ER in the prenatal hippocampus has not been reported but since hippocampal neurogenesis does not begin until E15-16 (Bayer, 1980) it is highly unlikely that cells in our transplants had differentiated into the ER phenotype. Our results using fetal transplants prior to the maturation of constituent cells into estrogen concentrating neurons not only demonstrates the presence of an intrinsic timetable for ER development but also suggests that neurons or their precursor cells are committed to an ER identity very early in development. Thus, a neuron's developmental fate for ER phenotypic expression appears to be specified before E14.

Although we observed a normal, region-specific pattern of ER development in intracerebral hippocampal, neocortical and possibly hypothalamic grafts, absolute ER levels in the transplants never reached their *in situ* age-matched levels. The transplant values at a theoretical donor age PND-7 were approximately 25% lower in neocortical grafts, 50% lower in hippocampal grafts, and 85% lower in HPOA grafts than ER concentrations which we previously reported in the intact PND-7 animal (O'Keefe and Handa, 1990). These data indicate that although the inherent profile of ER ontogeny is

intrinsically determined, maximum ER expression may require epigenetic signals. Interestingly, our homotopic transplants (i.e. frontal cortical grafts to a frontal cortical site in the host brain) contained a greater percent approximation in their ER content to normal levels than either of the heterotopic grafts. Similarly, when we extend this type of comparison to the heterotopic transplants, we also see a greater relative ER concentration in the hippocampal (a region of the cerebral cortex) grafts than in the HPOA transplants at 2 weeks post-grafting. Hence, placement into more appropriate target sites within the host brain are required for maximum level of ER synthesis or maintenance suggesting that appropriate access to target sites or other local environmental cues may be involved.

Few investigations have examined the developmental regulation of neural ER concentration. Although recent studies have demonstrated the down regulation of neural ER by estradiol in adult animals (Simerly and Young, 1991), it appears that gonadal steroids are not an absolute requirement for ER expression during development. Paden and McEwen (1985) found that ER's appear in fetal HPOA grafts transplanted to the choroidal pia overlying the superior colliculus independently of either the donor sex or the gonadal steroid environment of adult female hosts. These results support our conclusion that neural ER phenotypic expression is regionally specific and intrinsically

determined.

Our hypothesis that ER ontogeny is an autonomous, region-specific feature of neural tissues with optimal receptor levels influenced by environmental signals is supported by studies of other CNS receptors. Granholm et al.(1987) using a heterochronic in oculo grafting model to investigate the normal senescent alterations in cerebellar noradrenergic transmission, found that age-related reductions in the postsynaptic sensitivity of Purkinje cells appears to be intrinsically specified by the age of the grafted tissue. Similarly, beta-adrenoreceptor ontogeny in fetal neocortical and cerebellar transplants appears to be inherently determined by the graft site of origin while attainment of normal beta receptor levels may require a specific host milieu (Levin and Dunn-Meynell, 1989).

Several investigations have employed a variety of strategies in order to differentiate between the genetic and local environmental signals that contribute to the ultimate identity of neurons. Accordingly, there is substantial evidence that cortical precursor neurons are committed to a specific cell type and laminar fate before migrating to their ultimate position within the cortical plate. For example, x-irradiation of rat embryos causes cortical neurons to migrate abnormally and reside in ectopic locations but they display morphological features and connectivity patterns expected based on birth date not on

their abnormal position within the cortex (Jensen and Killackey, 1984). The abnormal cell migration in the reeler mouse mutant results in inverted cortical layers yet the electrophysiological properties and connectional patterns of the reeler's cortical neurons are normal for their birthdate (Caviness, 1976; Drager, 1981; Lemmon and Pearlman, 1981). Ventricular cells grafted from the primordial visual cortex of ferret embryos into homotypic sites within newborn hosts assume anatomical characteristics, positions, and connections which are predominantly donor-age specific (McConnell, 1985, 1988). Presumptive sensorimotor cortical tissue grafted homotopically into the sensorimotor region of the newborn rat neocortex responds to peripheral stimulation whereas heterotopic occipital transplants are unresponsive suggesting that the neurons were committed for their appropriate targets prior to transplantation (Castro et al., 1991). Barbe and Levitt (1991) utilized the expression of limbic associated membrane protein (LAMP), a specific phenotypic marker for limbic cortical neurons, in time-mismatched homotopic and heterotopic grafts to demonstrate that fetal neurons are committed to a cytoarchitectonic phenotype for LAMP expression by E14 in the rat. Neurons from E12 transplants, however, exhibited LAMP expression which was appropriate for the host site and hence dependent upon environmental signals. In these studies cells became phenotypically restricted between E12 and E14, prior to

their arrival at the cortical plate. These latter data are consistent with the ER phenotypic expression which we have reported herein. The possibility that transplants taken at an earlier developmental stage than that performed in this study might demonstrate a similar malleable response to environmental cues is intriguing.

In summary, we have begun to dissect the environmental and genetic factors required for ER phenotypic expression in the developing forebrain by employing a heterochronic intracerebral transplant paradigm. This is critical when considering the precisely coordinated sequence of events which must occur prior to the hormonal induction of CNS sexual differentiation. Our data show that the developmental decision for neocortical and hippocampal neurons to express the ER phenotype in an age-dependent fashion is made by embryonic day 14-15. This phenomenon may be similar for hypothalamic neurons as well. Thus, the region specific neural substrate ultimately required for estrogen action is determined early in embryonic life.

CHAPTER VI

DISCUSSION

Neurons are thought to express a growth program which undoubtedly involves sequential switching of genes for the ensuing array of developmental events. In these studies I have shown that the receptor for estrogen, an important neural growth molecule, changes in its expression during postnatal development of the rat hippocampal formation. Thus, ER ontogeny in the hippocampus and other brain regions may be regulated by the coordinated turning on and off of the ER gene. Moreover, the distinct temporal pattern of ER development appears to be preprogrammed or specified very early in embryonic life as demonstrated by the heterochronic transplantation study. When evaluated in their entirety, the findings from this dissertation demonstrate that the hippocampus is an important target for estrogen action during maturation of this limbic cortical structure.

The fundamental question(s) yet to be answered is what are the estrogen-induced cellular and molecular mechanisms which may underlie the sexual differentiation of the hippocampus and other brain regions during development? Estrogen's role in regulating cell growth and differentiation is much better characterized in peripheral

reproductive tissues. Only recently has there been direct experimental evidence for estrogen-mediated molecular events in neurons. Much of the following discussion is largely a theoretical scheme for estrogen orchestrated events during brain maturation with evidence provided from both non-neural and neural tissues.

Immediate-early genes (IEGs) or Third Messengers

Estrogen is well known for its mitogenic or DNA replicating properties in the peripheral reproductive tract. Several very early events that are important for estrogen's proliferative role in the uterus, and perhaps for its neurotrophic effects in the brain, is activation of genes which encode nuclear regulatory transcription factors (Murphy et al., 1986, 1987; Travers and Knowles, 1987; Loose-Mitchell et al., 1988; Weiscz and Bresciani, 1988; Weiscz et al., 1990). Transcription of most of these genes does not involve de novo protein synthesis; their products appear immediately upon stimulation of a cell (Morgan and Curran, 1989,1990). Therefore, these rapidly induced genes are often referred to as immediate-early genes (IEGs). A diverse range of extracellular signals such as external stimuli, growth factors, hormone or neurotransmitter receptors, and membrane depolarization with resultant ion fluxes elicit the expression of these genes (Curran and Morgan, 1985; Morgan and Curran, 1986; Aronin et al., 1990;

Cole et al., 1990). Their protein products then couple the stimulus to a cascade of gene transcriptional events and are thus called "third messengers" (Curran and Morgan, 1985).

One IEG product, Fos, is a nuclear phosphoprotein that can associate in transcriptional complexes with JUN, the product of the protooncogene c-jun, to generate a transcriptional factor heterodimer which binds to a DNA regulatory element termed the AP-1 binding site or jun/AP1 (Chiu et al., 1988; Curran and Franza, 1988). Estrogen has been shown to activate transcription from a jun/AP1-responsive test gene cloned upstream of a globin-promoter-chloramphenicol acetyltransferase (CAT) reporter gene (Weisz et al., 1990). The ability of estrogen to stimulate both c-fos and c-jun gene expression is not abolished by pharmacological blockade of protein synthesis, suggesting that the ER directly regulates promoter activity of these genes (Loose-Mitchell et al., 1988; Weiscz and Bresciani, 1988; Weiscz et al., 1990). Moreover, estrogen response-like elements (ERE) have been described upstream from the c-fos mRNA start site for transcription in several tissues (Greenberg et al., 1987; Weiscz and Rosales, 1990; Treisman, 1985). These observations putatively link estrogen and c-fos gene transcription by a direct ER interaction with c-fos enhancer elements.

Fos and Jun have been also implicated in the cascade of genomic events leading to prolonged functional alterations

in neurons such as the adaptive and plastic modifications seen, for example, in certain learning and memory processes (Morgan and Curran, 1990; Rose, 1991; Worley, 1990). Estrogen may induce the expression of these genes in both the developing and adult nervous system which might explain its profound morphological and functional effects in neural tissues. Indeed, estradiol administration to ovariectomized adult rats was found to increase the number of cells staining for the c-fos protein in the medial preoptic area, the ventromedial nucleus of the hypothalamus and the medial amygdala (Insel, 1990). Changes in c-fos protein immunoreactivity were not observed in the dentate gyrus granule cells or in the CA1 subfield of the hippocampus. However, Cattaneo and Maggi (1990), using slot-blot analyses, reported an elevation in c-fos mRNA in the murine hippocampus by 60 minutes following estradiol administration to ovariectomized adult females. Several explanations might account for the apparent discrepancies between the above two studies. First, the dose of estradiol used in the study by Cattaneo and Maggi (1990) was over 3 times that used by Insel (1990); this might indicate that the adult hippocampus is less sensitive than the hypothalamus or amygdala to estrogen-mediated induction of the protooncogene. This would not be surprising given the observation that there is a much lower ER concentration in this brain region in mature animals. Second, the antibody used for determination of the

c-fos protein recognizes other members of the c-fos protein family, including fos-B and fos-related antigen (Insel, 1990). Therefore, differential expression of members in this family might obscure detection of absolute amounts of c-fos in the hippocampus. Finally, elevated amounts of c-fos like protein in hypothalamic and other brain regions was not observed until 12 hours following estrogen treatment. The studies of Insel suggest that, under the conditions used in that study, estrogen stimulated c-fos by an indirect mechanism. Estrogen and other forms of activation rapidly induce c-fos mRNA and protein within the first hour of stimulation (Loose-Mitchell et al., 1988; Hunt et al., 1987). Despite the obvious need for more complete clarification of estrogen's role in hippocampal c-fos induction, the above findings add a new facet to our understanding of the ER-mediated cascade of events in the nervous system.

Another IEG transcription factor whose expression is increased in the uterus following estrogen exposure is zif268 (also referred to as NGF1-A) (Suva et al., 1991). Although estrogenic stimulation of zif268 has yet to be demonstrated in neural tissues, zif268 is regulated by nerve growth factor (NGF) in PC12 cells (Milbrandt, 1989), is present in the developing brain (Watson and Milbrandt, 1990), and its message is rapidly expressed in the adult rat hippocampus following N-methyl-D-aspartate (NMDA) specific

glutamate receptor subtype activation (Cole et al., 1989).

Activation of members of the IEG family, particularly c-fos, c-jun and zif268, is thought to be involved in synaptic plasticity such as that seen with long term potentiation (LTP) (Worley et al., 1990). LTP, the long-lasting enhancement of synaptic transmission following stimulation of glutamate receptors, may be a fundamental component of use-dependent modifications in synaptic efficiency underlying information storage (Collinridge et al., 1983; Collinridge, 1988). Expression of these genes is initiated, in part, by Ca^{2+} channel opening subsequent to glutamate receptor activation (Connor et al., 1988) and stimulation of the phosphatidyl inositol cycle resulting in phosphorylation events (Farooqui and Horrocks, 1991). All of the intervening intracellular signals and presumable genomic events are not known but what ultimately results is protein synthesis and morphological alterations in several synaptic parameters, including increases in dendritic spine density, diameter, synapse numbers and changes in the length of postsynaptic thickenings (Rose, 1991). These structural changes then mediate neurophysiological alterations such as LTP. It is intriguing to postulate that estrogen may promote neuronal growth and plasticity, in part, by stimulating transcription of the IEG family and thereby initiating the ensuing cascade of events. This interaction might provide a molecular basis for sex differences in

hippocampal morphology and circuitry underlying certain cognitive processes such as learning and memory function.

Polypeptide growth Factors

Polypeptide growth factors play a crucial role in the development and maintenance of neural circuits (Greene and Shooter, 1980; Snider and Johnson, 1989). In addition, growth factors may be important in the brain's response to injury and or in neurodegenerative disorders (Finklestein et al., 1988; Ernfors et al., 1989; Hefti et al., 1989; Tuszynski et al., 1990). Past studies have shown that estrogen increases the expression of several polypeptide growth factors which in turn have effects on the growth, differentiation and maturation of target tissues. For example, estrogen induces the expression of epidermal growth factor (EGF) and the EGF receptor and its mRNA in uterine tissue (Mukku and Stancel, 1985; Lingham et al., 1988). Moreover, EGF is intimately linked to and mimics estrogen's proliferative and growth functions in uterine tissue (Nelson et al., 1991). In the CNS, EGF has been shown to stimulate the proliferation and differentiation of glial cells (Leutz and Schachner, 1981; Simpson et al, 1982; Honegger and Guentert-Lauber, 1983; Almazan et al. 1985) as well as promote neurite outgrowth and survival in telencephalic neonatal rat neurons (Monnet-Tschudi and Honegger, 1989; Morrison et al., 1987). It is feasible that estrogen might increase the expression of EGF and/or its receptor in the

developing nervous system which would mediate or synergize with estrogen's organizational effects.

The insulin-related peptides are additional growth molecules which may interact with estrogen to promote neuronal differentiation and growth during development. Insulin-like growth factor I (IGF-I) and its receptor have been localized in the developing and adult mammalian nervous system (D'Arcole et al., 1984; Pomerance et al., 1988; Araujo et al., 1989; Werther et al., 1990; Bach et al., 1991). This peptide mediates most of the actions of growth hormone in the periphery. However, the presence of IGF-I and its mRNA in a variety of tissues including the brain has suggested that it may perform various paracrine or autocrine functions (D'Ercole et al., 1984). Region-specific temporal patterns of IGF-I gene expression have been reported in the developing rat brain (Bach et al., 1991). Interestingly, the cerebral cortex was found to transiently express IGF-I mRNA with maximal levels at postnatal day (PND) 8 and 13; in contrast, hypothalamic levels of this peptide increased from embryonic day 16 through PND-3 and remained elevated through adult life. These ontogenetic profiles are reminiscent of the unique temporal patterns seen for development of ER protein levels in the hippocampus and hypothalamus which I described in this dissertation. More tantalizing yet is the observation that estrogen interacts with the insulin-like growth factors in its neurite outgrowth promoting effects

(Toran-Allerand, 1989).

Nerve growth factor (NGF) is the prototype for molecules in the class of neural trophic polypeptide factors. Its mechanism of action has been the subject of intense investigation for several decades (Levi-Montalcini and Angeletti, 1968; Greene and Shooter, 1980; Halegoua et al., 1990). NGF affects several developmental parameters including neuron number, neuronal size, neurite outgrowth, dendritic arborization and neurotransmitter content and phenotypic expression in a wide variety of central and peripheral nervous system targets. In addition, the dependence on classical trophic factors in subsets of neurons in the peripheral and central nervous system involves unique critical periods of sensitivity (Thoenen et al., 1987). For example, cranial sensory neurons are dependent on trophic factors at the time which corresponds to the arrival of their axons at targets. The neurotrophic effects of NGF during critical developmental stages parallel those previously reported for estrogen-induced sexual differentiation in hormone sensitive neural substrates (reviewed in Toran-Allerand, 1984; Arnold and Gorski, 1984).

NGF has been linked to estrogen action in promoting neuronal survival and related developmental events during the process of sexual differentiation. For example, in the superior cervical ganglion (SCG) of rats, there is a greater number of neurons in males than in females due to the

organizing influence of neonatal estrogen (Wright and Smolen, 1983a, 1983b); this sex difference arises as a consequence of differential neuron death in the early postnatal period (Wright and Smolen, 1987). NGF content in the SCG and the pineal, one of its targets, is higher in males than females during the peak of naturally occurring cell death but before sex differences in neuron number have been found (Wright et al., 1987). One interpretation of these findings is that estrogen, which is present in higher concentrations in the male, might increase NGF levels, thereby promoting enhanced neuronal survival.

NGF may be also be subject to estrogenic regulation in hypothalamic tissues controlling sexual behavior (Yanase et al., 1988; Lara et al., 1990). Intraventricular infusion of NGF antiserum to estrogen treated neonatal female rats was found to prevent the normal estrogen-induced defemininization of adult copulatory behavior (Hasewaga et al., 1991).

A recent study has demonstrated sexually dimorphic expression of the NGF receptor (NGF-R) in the rat cholinergic septo-hippocampal system (Kornack et al., 1991). Female basal forebrain NGF-R mRNA levels were greater in females than in males during the first two postnatal weeks with levels rising sharply until postnatal day (PND)-7 and then exhibiting a plateau; receptor mRNA levels in males demonstrated a protracted and gradual developmental profile

so that by PND-21 levels were not different between the sexes. The alterations seen were similar to previous reports of the more rapid development of the acetylcholine biosynthetic enzyme, choline acetyltransferase (CAT), in female than in male neonatal rats (Loy and Sheldon, 1987). NGF is synthesized in the hippocampus, binds to receptors on afferent terminals, is retrogradely transported to basal forebrain cell bodies and is known to increase CAT activity (Gnahn et al., 1983; Ayer-LeLievre et al., 1988; Johnson et al., 1987; Williams and Rylett, 1990). Thus, differential NGF-R expression in males and females, perhaps induced by differential perinatal exposure to estrogen, may explain the sexually dimorphic development of CAT in the septo-hippocampal system.

Only recently has the mechanism for gender differences in the expression of neurotrophic factors and their respective receptors been directly investigated. Several neuronal subsets in the developing rodent CNS, including neurons in the hippocampus and cortical subplate, have been found to coexpress the mRNAs and protein for the ER and NGF-R, as well as those for NGF and BDNF (Miranda et al., 1991). These observations lend credence to a putative autocrine or paracrine role for estrogen and nerve growth factors in the developing hippocampus and cerebral cortex which may contribute to or mediate estrogen-induced neuronal growth, differentiation and survival in these and other brain

regions. It is plausible that estrogen regulates the production of NGF-R which might provide a mechanism for estrogen's trophic effects. In fact, ovariectomy in adult female rats dramatically reduces NGF-R mRNA expression in dorsal root ganglion neurons, a classical NGF peripheral target that also contains ER mRNA (Sohrabji et al., 1991). Thus, estrogen appears to up-regulate NGF-R mRNA. Increased binding sites for NGF had previously been shown in the SCG and DRGs of early postnatal female rats following estrogen treatment (Wright et al., 1988). In addition, exposure of NGF to pheochromocytoma (PC12) cells upregulates ER mRNA levels in the now-differentiated cell (Sohrabji et al., 1991). Simultaneous estrogen and NGF administration to embryonic septal/diagonal band explants increases the number of cells expressing ER mRNA as well as enhances ER mRNA levels in individual neurons (Toran-Allerand, et al., 1991). It appears, then, that there is a dual synergistic interaction between estrogen and NGF with either ligand enhancing neuronal sensitivity to the other by upregulating receptor expression.

This is an exciting, relatively unexplored area of research. Future advances in this arena may prove to be fruitful for more fully characterizing the interactions between neural growth factors and estrogen and their respective receptors and hence, in understanding the molecular mechanisms underlying brain sexual

differentiation.

Neural Development Associated Proteins

A number of proteins are instrumental for the differentiation and growth of axons and dendrites. Estrogen may promote neuronal growth in a more direct manner than that discussed above for the IEGs and growth factors by regulating the expression of these "neural development associated proteins".

Axonal growth and synaptogenesis involves the synthesis of several proteins in the growth cone. Growth-associated protein 43 kDa (Gap-43) is one of these proteins. It is a rapidly transported, membrane-bound phosphoprotein whose induction is correlated with axonal growth, regeneration and plasticity (Meiri, et al., 1986; Benowitz and Routtenberg, 1987; Skene, 1989). High levels of GAP-43 mRNA are found in the neonatal cerebral cortex (Karns et al., 1987) and in regions of the adult brain which are thought to be highly plastic such as the hippocampus and the association cortices (Benowitz et al., 1988). Moreover, phosphorylation of GAP-43 is correlated with long-term potentiation in the rat hippocampus (Lovinger et al., 1985) and with passive avoidance task training in chicks (Rose, 1991), findings which implicate GAP-43 in the synaptic plasticity associated with memory formation (McNaughton and Morris, 1987).

Estrogen administration to gonadectomized male and female adult rats substantially increases GAP-43 mRNA levels

in the ventromedial nucleus of the hypothalamus (Lustig et al., 1991). In addition, this nucleus, as well as cerebral cortical tissue, displays a sexual dimorphism in steady-state GAP-43 mRNA levels with higher levels in males than females. Differential GAP-43 mRNA levels between the sexes was not dependent on adult circulating gonadal hormones levels. The implications from this study are that estrogen's enhancement of neurite outgrowth may be mediated in part by its positive regulation of GAP-43 expression. Moreover, demonstration of the adult sex dimorphism in GAP-43 mRNA levels suggests that perinatal estrogen may play a role in establishing this sex differences. Thus, regions of the cerebral cortex and the hippocampus may respond to endogenous estrogens during the early postnatal period, when ER levels are high in these tissues, by increasing GAP-43 levels and ultimately affect neuronal process formation. This could provide a molecular basis for the known sex differences in neural circuitry and plasticity in the hippocampus and cerebral cortex. It is intriguing to postulate that gender differences in learning and memory functions mediated by the hippocampus may be brought about, in part, by differential hippocampal GAP-43 expression between the sexes, as was found in the cerebral cortex and the hypothalamus.

Interestingly, NGF induces GAP-43 protein expression in PC12 cell lines (Basi et al., 1987; Benowitz and

Routtenberg, 1987; Karns et al., 1987) and PC12 cells transfected with the human GAP-43 gene demonstrate accelerated neurite outgrowth and enhanced sensitivity to NGF (Yanker et al., 1990). The rat PC12 cell line differentiates into the neuronal phenotype in response to NGF and it is a cell culture system commonly utilized for studying neuronal differentiation (Basi et al., 1987). Thus, estrogen and NGF demonstrate similar effects on enhancement of GAP-43, which are likely to be responsible, at least in part, for their growth promoting effects. Alternatively, estrogen may modulate NGF levels and or sensitivity of neurons to NGF, thereby altering GAP-43 expression and ultimately neuronal outgrowth.

Microtubule assembly is a key event in neurite elongation and neuronal process formation. Microtubule-associated proteins (MAPs) are intimately involved in this process and promote tubulin polymerization and microtubule stability during process extension (Tucker, 1990). Tau is a MAP which is segregated primarily in the axonal compartment of mammalian neurons (Binder et al., 1985). Furthermore, tau plays a role in inducing neuronal polarity and in axonal differentiation (Caceras and Kosik, 1990). Estrogen has been shown to increase tau protein levels in dissociated cultures of hypothalamic neurons; this affect precedes and accompanies the enhancement in microtubule stabilization and neurite elongation seen in response to this hormone

(Ferreira and Caceras, 1991). Hence, estrogen may regulate axonal growth and differentiation by enhancing tau expression.

Intracellular calcium

Intracellular calcium (Ca^{2+}) concentration is another important mediator of i) neuronal growth during development and plasticity (Kater et al., 1988), ii) neurotransmitter release and synaptic efficacy (Augustine et al., 1987), and iii) neurodegeneration associated with excitatory amino acid neurotoxicity (Siesjo, B.K. et al., 1989). Intracellular calcium is a key regulator of the neuronal growth cone (Kater et al., 1988) and it has been implicated in the outgrowth-regulating and neuro-protective functions of growth factor molecules (Mattson et al., 1989; Mattson, 1990; Cheng and Mattson, 1992).

Estradiol has been shown to suppress Na^{+}-dependent Ca^{2+} efflux (also known as the Na^{+}-Ca^{2+} exchanger which is a plasma membrane calcium extrusion mechanism) from rat brain synaptosomes from several brain areas including the hippocampus (Horvat et al., 1991), suggesting an estrogenic modulation of neuronal calcium homeostasis. This finding may suggest an additional mechanistic role for estrogen's effect in promoting neuronal growth (Blanco et al., 1990), plasticity (Gould et al., 1990; Woolley et al., 1990) and seizure susceptibility (Buterbaugh and Hudson, 1991) in the

hippocampus.

Conclusion

Although, it is very difficult at this time to temporally link ER expression with discrete developmental events in the hippocampus, given that the individual hippocampal neurons expressing the ER during perinatal life have yet to be defined, the preceding observations unfold a potential foundation for estrogen's role in sexual differentiation of the hippocampus. Recruitment of several immediate early genes with their ensuing cascade of intervening intracellular signals is a likely molecular event following ER activation. Estrogen action in neuronal development also undoubtedly involves complex interactions with growth factors which may secondarily or synergistically result in enhanced neuronal differentiation and growth. In addition, estrogen may directly alter neuronal Ca^{2+} homeostasis and the expression of molecules such as GAP-43 and tau which are instrumental for process formation, growth and plasticity. Future studies clarifying the molecular array of events and signal transduction mechanisms following ER activation in neural tissues will provide crucial insights for how differences between the male and female brain and ultimately, sexually dimorphic behavior patterns, arise.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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