1990

The trophic effects of estradiol on virally transformed hypothalamic cell lines

Jane Elliott Rasmussen
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Date

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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by
Jane Elliott Rasmussen
1990

ABSTRACT

THE TROPHIC EFFECTS OF ESTRADIOL ON VIRALLY TRANSFORMED HYPOTHALAMIC CELL LINES

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Although it appears that the perinatal development of sexual phenotype in the rodent brain is determined by exposure to estradiol, generated locally via aromatization of androgen, the mechanisms underlying this process are not fully understood. The development of an in vitro model of hormone action based upon examining the effects of sex steroids on estrogen-sensitive fetal rat hypothalamic cells could provide important information. Therefore, using serum-free, growth factor-deficient conditions the effects of 17 alpha- and 17 beta-estradiol, testosterone, 5 alpha-dihydrotestosterone (DHT), and tamoxifen on survival of two SV-40 transformed estrogen-receptor positive rat hypothalamic cell lines were examined, with the hope of establishing an in-vitro model of sexual differentiation of the rat hypothalamus. In one cell line, designated RCF-8, both 17 beta-estradiol and testosterone significantly increased cell survival at picomolar concentrations. The

effects of both estradiol and testosterone were blocked by tamoxifen, and they could not be reproduced by the non-aromatizable androgen, 5 alpha-dihydrotestosterone or the inactive estradiol isomer, 17 alpha-estradiol. In the other cell line, RCA-6, addition of 17 beta-estradiol led to inhibition of cellular proliferation, which was reversed by the addition of tamoxifen. In an estrogen receptor-negative hypothalamic cell line, RCF-12, estradiol had no net effect on the growth pattern.

After establishing the properties of this model cell line, RCF-8, the effects of estradiol treatment on the response to endogenous hypothalamic growth factors was examined. For these studies we chose insulin and insulin-like growth factor I (IGF-I). We found that estradiol induces competence such that RCF-8 cells have a mitogenic respond to IGF-I, but not to insulin.

In summary, the estrogen binding capacity and the responsiveness to physiologic concentrations of estradiol and testosterone, but not DHT, make the RCF-8 cell line a potential in vitro model of hypothalamic sexual differentiation and through the use of this estrogen-sensitive hypothalamic cell line model, we have a unique opportunity for studying the cellular mechanisms underlying sexual dimorphism.

ACKNOWLEDGEMENTS

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DEDICATION

To Drs. Howard and Jane Spence Rasmussen

who believed in me even when I didn´t believe in myself,

Thanks.

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INTRODUCTION

Sexual dimorphism is obvious in most mammalian species because of the differential development of internal and external genitalia as well as extragenital features such as body size. The term "sexual dimorphism" refers to any differences in form regardless of whether they are manifest at the behavioral, morphologic or molecular level. In the central nervous system, sexual differentiation is present in many vertebrate species, including hamster, mouse, rat, various bird species, as well as human beings (1-5). Divergence occurs both structurally, in the size of specific brain nuclei, neuronal morphology and in synaptic networks (i.e. patterns of neuronal connectivity and dendritic branching patterns), as well as functionally in sex specific behavioral patterns, regulation of food intake and body weight, and reproductive physiology (6-11). There are three well-characterized areas in the central nervous system, that show marked anatomical differences between the sexes: the sexually dimorphic nucleus of the pre-optic area in the rat, the song nuclei in the zebra finch and the canary, and the spinal nucleus of the bulbocavernosus in the rat spinal cord. In each of these systems nuclear volume and cell number are higher in the male.

The concept of sexual dimorphism of the brain implies

that the many functional and structural differences evident in mammalian brain are not due to autonomous sex differences but rather are imposed on an inherently female or at a minimum bipotential brain during development. The aspects of brain function characteristic of the male are manifest in the developing brain through the action of hormones at a critical period in development (12). For example, human embryos of both sexes develop in an identical fashion for the first two months of gestation, and only thereafter do anatomical and physiological development diverge to result in the formation of the male and female phenotypes. The period of greatest sensitivity to gonadal hormones in terms of sexual differentiation varies with the developmental state of the organism at birth (i.e., in some species hormones act prenatally; in others perinatally and in some, hypothetically, could act postnatally). It should be mentioned that separate functional systems in the central nervous system may differentiate independently both in terms of the period of development and in qualitative and quantitative hormonal sensitivity, i.e. the critical period of development of one hypothalamic nuclei or analogous system, may occur prior to differentiation of another and that the concentration of hormone needed to elicit the change in one system may differ from another.

The basic experimental approach that has been used to

document the concept of sex-steroid brain sexual differentiation has been to modify the hormonal environment perinatally. This is generally accomplished by exposing embryonic/fetal females to exogenous androgenic steroids or by preventing exposure of the perinatal male to endogenous testicular hormones by gonadectomy or drug treatments. Subsequently, physiological, behavioral or anatomical differences are then sought in adulthood.

The initial reports of sex-specific brain differences appeared in the late 1920's and early 1930's. The first noteworthy experiments concerning sexual differences in the brain were performed by Pfeiffer (13) over 50 years ago. In an elegant set of experiments, he showed that the expression of masculine patterns of pituitary gonadotropin secretion in adulthood was dependent on factors released from the testes during early postnatal life. Thus, the development of masculine patterns of gonadotropin secretion could be induced in genetic females by transplantation of a testis shortly after birth; whereas castration of genetic males at birth resulted in the development of the characteristically female patterns of gonadotropin release.

Alfred Jost between 1947 and 1952 is credited with the elucidation of the fundamental mechanism of sexual dimorphism. The Jost formulation states that sexual differentiation is a sequential, ordered and relatively simple process. Chromosomal sex directs the development of

either ovaries or testes. If testes develop, the hormonal secretions elicit the development of the male phenotype. If the ovaries develop or if no gonad is present, anatomical development is female. Thus, whatever the mechanisms by which chromosomal or genetic sex is translated into gonadal sex, it is the action of the gonads as endocrine organs that is responsible for the development of the sexual phenotypes.

One of the most divergent brain systems is the sexually dimorphic nucleus of the preoptic area (SDN-POA) of the rat hypothalamus in which anatomical differences are so marked that one can sex the brain with the unaided eye after thionin-staining. Within the last fifteen years using the rat hypothalamus as their model, Gorski and co-workers (14-16) have carefully shown that treatment of female rats with testosterone propionate injections from day 16 gestation through the first ten days of postnatal life produces an irreversible and permanent masculinization of the brain as documented by lordosis reflexes, receptivity and mounting behavior, as well as the striking anatomical differences in the preoptic area. In male rats the volume of the SDN-POA is 5-7 times greater than that of the female. Since neuronal volume is not increased, this means that the number of neurons comprising the nucleus in the male is significantly greater than in the female. In addition, Jacobson et. al. (17), again using the SDN-POA, were able

to show that gonadectomy of male fetuses neonatally has equally permanent effects, leading to genetic males with "female" brains, (i.e. male fetuses which underwent castration showed no specific increase in volume of the SDN-POA).

As it was established that differences in the hormonal environment could lead to anatomical changes in the rat brain, the next problem was to determine which hormone or hormones were influencing the cells in these areas. A number of possibilities needed consideration. Since testosterone treatment can essentially substitute for the testes in inducing masculine patterns of gonadotropin release and behavior (18), it seemed likely that either testosterone, aromatized metabolites of testosterone such as estradiol, or reduced metabolites of testosterone such as 5 alpha-dihydrotestosterone were the responsible agent(s). In 1972, Davidson and Levine (19) suggested that the "future masculinization of the reproductive behavioral pattern is the result of the action of androgen at a critical period in early development. In the absence of circulating androgen, the brain remains in its neutral state which is one of potential femaleness". This hypothesis assumes that under normal physiological circumstances, during the critical period of development there must be a high concentration of androgen in the circulation of the male, but an absence of androgen in the

female animal. It is premature, however, to conclude that sexual dimorphic phenomenon in the brain are simply a function of the presence in male and absence in females of unbound circulating testosterone. Radioimmunoassay measurements have shown that in the rat, androgens circulate in females as well as in males during the critical period when sexual differentiation of the central nervous system occurs. The actual amounts of androgen necessary for brain dimorphism still remains controversial. Some reports have demonstrated consistently higher testosterone concentrations in males than in females during early post-natal life (20), whereas others have shown a considerable overlap in the amounts present in the two sexes (21).

During the 1940's several workers reported the surprising fact that treatment of female rats with high doses of estrogen prior to or shortly after birth resulted in a pattern of anovulatory sterility in adulthood which closely resembled that observed after perinatal testosterone administration (22-24). Subsequent work confirmed and extended this finding; many of the effects of testosterone on the developing brain could be reproduced by estrogen (25).

In the early 1970's it was well known that androgens, which were made by the gonad or adrenal gland could be converted by an aromatase enzyme elsewhere (peripheral

conversion), furnishing a considerable portion of the daily production of estrogens (26). It therefore seemed likely that similar metabolic transformations could occur in the central nervous system. The aromatase hypothesis (27), proposing that estrogens formed in or near their target cells from androgen precursors could account for the action of testosterone in certain neuroendocrine processes such as brain differentiation, was developed conceptually to account for the above findings. The central tenet of this hypothesis is that estrogen is produced locally via aromatization and acts as a regulatory agent. This hypothesis was satisfying for several reasons: local production of the hormone 1) avoids unnecessary exposure of the entire body to active hormone metabolites on their way from a distant producing cell; 2) it avoids the need for large amounts of product to overcome dilution in the blood stream; and 3) it provides a localized feedback loop for efficient control of the estrogen effects. The full impact of the above work and subsequent aromatase hypothesis did not become apparent until the early to mid 1970's when three observations firmly established the role of estradiol in the process of sexual dimorphism. First, 5 alpha-dihydrotestosterone (DHT) and other ring A reduced androgens were shown to be far less effective than either testosterone or estradiol at inducing defeminization of the neonatal rat brain (28-30). Secondly, the effects of

testosterone treatment in neonatal female rats could be blocked by estrogen antagonists (31). Finally, the developing brain, like many peripheral tissues was found to be a site of androgen to estrogen conversion (32). The above findings clearly establish that exposure of the developing brain to estrogen effects mammalian sexual development in much the same way as early exposure to testosterone.

In placental mammals, the fetus is continually exposed to endogenous estrogen from the placenta and maternal circulation. If estrogen formation within the brain plays a vital role in sexual differentiation, then it follows that the fetal brain must somehow be protected from the influence of high effective circulating estrogen levels. In rats and mice the mechanisms of this protection are well established. In each of these species, the immature brain is functionally denied circulating estrogen by a plasma estrogen binding system. The developing yolk sac and fetal liver synthesize an estrogen binding protein (fetoneonatal estrogen binding protein, or FEBP) which circulates at high concentrations during the latter part of gestation and then gradually disappears over the first few weeks of postnatal life (33-34). This protein, which is immunochemically indistinguishable from the plasma alpha-globulin, alpha-fetoprotein, binds and effectively sequesters virtually all of the estrogen present in the fetal and neonatal

circulations (8,35). Significantly, it binds testosterone poorly: hence testosterone is free to enter the brain where it can be converted locally to estradiol via aromatization.

Several experimental observations have confirmed the effectiveness of the FEBP protection mechanism. Although the levels of estradiol in the blood of the neonatal rat, both male and female, are high, free estradiol does not seem to be readily available in the tissues (36-37). The administration of antibodies to alpha-fetoprotein in newborn rats produces effects on sexual development that resemble those of estradiol or testosterone injections (38).

It is not possible to extend the FEBP binding hypothesis species other than rats and mice as of yet. Although alpha-fetoprotein is present in many vertebrate species, the ability to bind estrogen seems to be property of the particular species-specific alpha-fetoprotein. As we are able to detect similar sexual dimorphic phenomenon in species other than rat, including man, there must exist an as of yet undetermined alternative protection system. There is evidence, however, suggesting that the aromatase hypothesis may be important in species other than rat, such as guinea pigs and humans (27).

Once estrogen is formed locally in the brain how does it modulate the final number of neurons in the SDN-POA? Gorski, as well as other investigators (8,35,39) have

postulated several mechanisms, not necessarily mutually exclusive, including: 1) estrogens may stimulate or prolong neurogenesis; 2) estrogens may influence the migration of neurons from their origin in the ependymal lining of the third ventricle to the region of the SDN-POA; 3) in the vicinity of the SDN-POA estrogen may promote the aggregation of these neurons into the distinct nucleus, perhaps by altering a cell surface recognition factor; 4) estradiol may promote neuronal survival during the phase of programmed neuronal death; and 5) estradiol may influence the specification of neurons destined to form the SDN-POA, that is, by activating or suppressing certain genes, estradiol determines the functional specificity and thus perhaps the migration, aggregation and even survival of more neurons in the male.

Although, the exact cellular mechanism(s) by which estrogen ultimately increases the final number of SDN-POA neurons remain to be elucidated, estrogenic regulation of other cell types in vivo, and in vitro provides important insights into the way the process may occur in the central nervous system (40-45). Direct interactions with estrogen receptor positive target cells and indirect interactions via production or modulation of local growth factors to increase cellular proliferation have been reported (46-48). It seems reasonable to suppose that similar mechanisms may underlie the developmental effects of estrogens on the

central nervous system. The most widely accepted hypothesis (43,44,49-52) for estradiol´s action advocates a direct estrogenic effect. In this view, estradiol binds to a specific nuclear or cytoplasmic receptor in the representative cell (including neuronal cell types), followed by translocation of the complex to the cell nucleus and initiation of the cellular response(s) (9). The newer appreciation of the role of local growth factors in brain formation raises the question about indirect estrogen effects mediated by such growth factors.

During the development of the mammalian brain many neurons are formed which never survive to adulthood. The inability of many neurons to obtain sufficient support from local neurotropic factors leads to their death. This overproduction of neural precursor cells and competition for limited neurotrophic support, termed "programmed cell death", occurs in both the central nervous system as well as in the peripheral nervous system (53-54).

Experimental models of neurotrophic influences need to attempt to recreate this pattern of selective survival, furnishing systems with limited support and declining cell number. The prototypical neurotrophic factor is nerve growth factor (NGF). Many investigators have documented NGF´s neurotrophic effects using model systems (55-58). For many years, NGF was believed to be the only neurotrophic factor. Recent work from numerous

investigators (58) has shown that many other ubiquitous growth factors need to be considered as neurotrophic factors. The experimental element limiting the identification of other neurotrophic factors is that defined neuronal culture systems are difficult to establish. These systems are necessary so that the activity of putative trophic agents can be quantified from data on the ability to support neuronal survival.

The somatomedins or insulin-like growth factors, constitute a family of peptide growth factors which are structurally and evolutionarily related, possess insulin-like activity, and have mitogenic effects in a wide variety of cell types. Two major human somatomedins have been purified and sequenced, Insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II). Insulin-like growth factor I is a basic peptide of 70 amino acids with a molecular weight of 7649. Structurally, the adult form is a single polypeptide chain with 3 disulfide bridges, having strong structural homology with human proinsulin. It is synthesized and secreted de novo in many tissues, including liver, kidney, testes, submaxillary gland, pancreas, heart and nervous system. The highest levels are found in the liver and the brain. In addition, there is a fetal form which varies somewhat in structure from the adult form, having a truncated amino-terminal region.

There has been considerable controversy concerning the presence of IGF-I in the brain (59-60). Much of the confusion resulted because it was not recognized initially that a fetal brain form of IGF-I existed and researchers were unable to detect IGF-I immunoreactivity in the adult brain (61). The existence of a fetal form was proposed by Sara et. al. (62) based on the presence of fetal tissue somatomedin receptors, and somatomedin growth-promoting activity on fetal cells in vitro (63-64) despite low or undetectable levels of immunoreactive adult IGF-I or IGF-II in fetal serum. The presence of the fetal form of IGF-I was proven by utilizing a radioligand receptor assay utilizing human fetal brain plasma membrane receptor as the matrix and IGF-I as the ligand (62). This method revealed elevated levels of somatomedins (not attributed to either immunoreactive IGF-I or IGF-II) in the fetal circulation in levels four times that seen in adult. The fetal form of IGF-I is thought to be unique to the central nervous system found only in the fetal and early neonatal animal. The levels in brain tissue slowly decline after birth to nondetectable levels in the adult. A transition from production of fetal to adult forms of somatomedins at about the time of birth has also been proposed (62). It is noteworthy that in healthy newborns, a significant correlation was found between fetal brain serum somatomedin levels and birth weight and length (62). This finding

suggests that embryonic/fetal IGF-I may play an active role in the regulation of fetal brain development and growth and perhaps in early postnatal brain growth. We postulate that estrogens may act to increase the survival of certain neurons by interacting with local factors such as IGF-I.

A problem in testing this hypothesis, however, is that the substrate on which steroids act in the developing brain consists of a heterogeneous population of cells, only a small proportion of which contain estrogen receptors. In the normal brain, responses to estrogen action occur against a background of developmental events in estrogen-insensitive neuronal and glial systems that may mask the specific effects of the hormone. Even where a response to estrogen is observed, it may be difficult to determine whether it is due to direct or indirect actions of the hormone, since effects on the primary steroid target cell may be transmitted to many other cells through changes in the release of neurotransmitters or neuromodulators by other cells or by the target cell itself.

Because of the complexity of intracellular signalling in the developing central nervous system, we have attempted to examine the effects of estrogen on the survival of a single isolated neuronal precursor cell. Dr. Robbins' laboratory has transformed millions of fetal hypothalamic precursor cells by infection with simian virus 40 (SV-40). The resultant cell lines were screened for the presence of

numerous specific neuronal antigens, glial antigens and estrogen receptors.

By the use of such immortalized cell lines, we were able to examine the action of a specific hormone (e.g. estradiol) on a single cell type. We were interested in: 1) identifying a hypothalamic cell line that could specifically bind estradiol (i.e. possesses estrogen receptors). 2) show increased cell growth or survival of an estrogen-receptor positive cell line as a direct result of estradiol action, 3) demonstrate aromatase activity by inducing a growth response of the cells when exposed to testosterone, and 5) induce no growth increase upon exposure of the cell line to 5 alpha-dihydrotestosterone.

Upon identifying such a cell line, we were interested in exploring the possible mechanism(s) of action for estrogen in sexual dimorphism. Specifically, we were interested in exploring whether the estradiol effect of increasing cell number could be due to modulation of the action of another local hormone or tissue growth factor. Because of evidence suggesting the presence of a unique fetal brain form of insulin-like growth factor I activity this is where we focused our attention. Using our model cell line, we examined the effects of treatment with 1) estradiol and IGF-I alone and in combination; and 2) estradiol and insulin alone and in combination.

MATERIALS AND METHODS

Materials

SV-40 virus (B381, $2x10^8$ pfu/ml) was a gift from Dr. J. Cook (National Asthma Center, Denver CO.). All steroids and other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO.). There was no further purification of these substances before use. ^{3}H- 17 beta-estradiol (114.4 Ci/mmol) was purchased from New England Nuclear. Estrogen-deficient gelded horse serum was purchased from the Lowell Crowther Ranch (P.O. Box #8 Sanford CO. 81151). Recombinant insulin-like growth factor I was purchased from Bachem (Torrance, CA.). Purified Porcine insulin was obtained from Sigma (St. Louis, MO.).

Preparation of the transformed cell lines

The cell lines coded RCF-8, RCF-12 and RCA-6 were obtained from rat fetal hypothalamus (embryonic day 16) irrespective of genetic sex, and were virally transformed using SV-40 virus according to methodology reported previously (65). Briefly: Rat embryos were removed by Cesarean section from timed pregnant Sprague-Dawley rats and their brains dissected. The basal diencephalon from each embryo was isolated and pooled together. The pooled

diencephalon were dispersed by gentle trituration in a 0.01% papain- 0.1% neutral protease- 0.01% deoxyribonuclease solution. The resultant cell suspension was washed and resuspended in Earle´s minimal essential medium (MEM,Gibco) containing 10% fetal calf serum (FCS). The cells were counted using a hemocytometer. Cells ($4x10^6$) were incubated for 3 hours at 37°C with $5x10^7$ pfu of SV-40 virus, a final concentration of 12.5 pfu/cell. After virus absorption, the cells were plated in 25 cm^2 flasks, and observed for development of transformed foci. Plating medium was Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 5mM glucose and 10% FCS. Foci of rapidly dividing cells were collected by trypsinization and diluted to a concentration of 0.1 cell/ml and then plated in microtiter plates in the same medium. This process of limit dilution was used to ensure that each cell line originated from a single cell. Wells which contained confluent cell layers were then trypsinized, rediluted, as before, and then replated. All of the cell lines were found to express the SV-40 large T antigen, determined by the immuno-fluorescent method of Pope et. al. (66). In addition, these cell lines were found to express both neural and glial markers.

We have noted that RCF-8 cells older than 25 passages occasionally develop an increased basal growth rate simultaneous with a loss of estrogen responsiveness. The

normal phenotype is recovered by thawing cells stored in liquid nitrogen from very early passages. In one case we sub-cloned the RCF-8 cells by limit dilution and were able to recover many cell lines which retained the estrogen responsiveness of the parental line.

Cell culture

Stock cultures of the cell lines were routinely grown in 75 cm^2 flasks containing MEM supplemented with 10% FCS, 2 units/ml penicillin (Gibco), 2 units/ml streptomycin (Gibco) and 33mM dextrose. The cells were grown at 37°C in 5% carbon dioxide-95% humidified air as monolayer cultures and were subcultured at weekly intervals.

Cells from subconfluent stock flasks were suspended by treatment in 0.25% trypsin/2mM EDTA at pH 7.6, washed, resuspended in serum-free media, counted, and then plated in multiwell polystyrene dishes (35mm) at an initial density of 10^5 cells/well. Passages between 10 and 27 for RCF-8, between 10 and 69 for RCF-12 and between 10 and 73 for RCA-6 were used for experiments. Cells were cultured in 2 ml. of DMEM-Ham's F-12 (1:1) without phenol red, supplemented with 1.2gm/liter $NaHCO_3$, 15mM HEPES, 0.1mg/ml transferrin, 10^{-5}M putrescine, $2x10^{-8}$M progesterone, 10^{-7}M corticosterone, 10^{-5}M triiodothyronine, 1ug/ml arachidonic acid, and $2x10^{-8}$M Na_2SeO_3 (67). The pH indicator phenol red was eliminated from the experimental media because of its

reported estrogenic properties (68). The steroids were added from stock solutions in ethanol so that final concentrations of ethanol were less than 0.01%. Test peptides were diluted in culture medium from a stock solution just before use. Cultures were maintained for 7 days. At the end of this time, adherent cells were resuspended by trypsinization and counted using a hemocytometer. Unless otherwise indicated, in every experiment all treatments were done in triplicate.

Throughout the week in culture the number of viable cells (x 10^4) present after plating 10^5 RCF-8 cells at several time points was: day 2, control 1.65 $\pm$ 0. 23, estradiol (1 pM) 3.25 $\pm$ 0.50; day 4, control 1.28 $\pm$ 0.15, estradiol 2.53 $\pm$ 0.23; day 6, control 0.86 $\pm$ 0.11, estradiol 2.11 $\pm$ 0.17 (triplicate experiments, total n=12 for each point).

Binding studies

Cell lines obtained by the method outlined above were screened for the ability to bind 17 beta-estradiol using a modification of a well characterized ^{3}H-estrogen nuclear binding assay (69). Briefly: 24 hours prior to each study, cells from stock cultures, confluent T75 flasks, were transferred to medium containing gelded horse serum. At t=0, ^{3}H-estradiol was added to each flask to a final concentration of $2x10^{-9}$M. Control flasks for non-specific binding received the isotope and excess ($1x10^{-7}$M)

diethylstilbestrol (DES). The flasks were incubated at 37°C for 1 hr. and then put on ice. The medium was removed and the cell layer scraped off using a rubber policeman. The cells were homogenized in a buffer containing 0.32 M sucrose, 3mM $MgCl_2$, 1mM potassium phosphate, 0.025% Triton-X 100, pH 6.5 and the nuclei isolated by centrifugation at 850g for 10 minutes. The nuclear pellet was washed twice in the above buffer without Triton-X 100 and then extracted overnight by incubation with ethanol. The ethanol extracts were counted in Opti-fluor and the DNA in the extracted pellets was assayed by the method of Burton (70). Results of the binding studies were expressed as amount (fmol) of specific nuclear binding per mg of DNA. Specific binding was calculated as the difference between the results in the presence and absence of DES. All binding studies were done in at least triplicate.

RESULTS

Estrogen Binding Studies

Four of fifteen screened rat hypothalamic cell lines (RCF-8, RCA-6, RCF-17 and RCD-15) displayed measurable estradiol binding (>5 fmol/mg DNA). The highest specific binding (132.1 fmol/mg DNA) occurred in the RCF-8 cell line. The next highest level (23.1 fmol/mg DNA) was present in the RCA-6 cell line. The other two lines that displayed measurable binding, RCF-17 and RCD-15, had values of 21.5 fmol/mg DNA and 7.82 fmol/mg DNA, respectively. Eleven cell lines, including RCF-12, exhibited no specific binding in this assay.

Effects Of Estrogen On Cell Growth

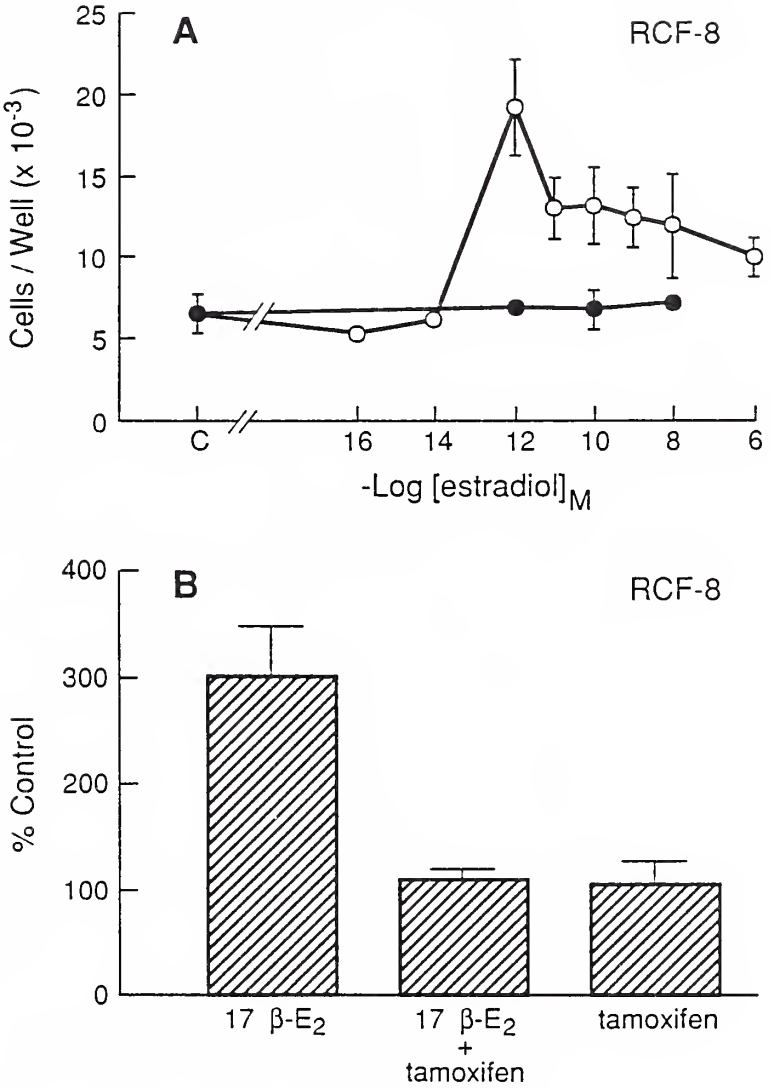
RCF-8 cells were growth arrested by plating at a low density in a serum-free medium (see methods). By day 7 in vitro, 10^5 RCF-8 cells had declined to 0.63 $\pm$ 0.21 x 10^4 viable cells per well (mean $\pm$ SD, n=78). The effects of 17 beta-estradiol and 17 alpha-estradiol on the survival of these cells, under these conditions are depicted in Figure 1A. 17 beta-estradiol, at every concentration tested between 1 pM and 1 uM significantly ($p< 0.025$) increased the number of viable cells present on day seven. The maximal response was observed with 1 pM 17 beta-estradiol,

which resulted in a more than three-fold increase in surviving cells (1.91 $\pm$ 0.29 x 10^4 viable cells; n=30, $p<0.01$). Higher concentrations of 17 beta-estradiol (up to 1 uM) also significantly increased cell survival, but the magnitude of the effect progressively declined as the concentration increased. Neither 0.1 nor 10 fM 17 beta-estradiol increased the survival of RCF-8 cells in this paradigm. 17 alpha-estradiol did not increase cell survival at any concentration tested. Estriol (100 pM) also significantly ($p<0.05$) increased the number of surviving cells on day seven (control 0.98 $\pm$ 2.14 x 10^4; treated 1.84 $\pm$ 0.34 x 10^4; mean $\pm$ SD, n=9). The enhanced cell survival induced by 1 pM 17 beta-estradiol was completely reversed by the addition of 100 pM tamoxifen (Figure 1B) although tamoxifen alone (100 pM) had no effect on cell survival. No morphologic changes were noted on exposure to the above agents.

Figure 1A: Effects of 17 beta-estradiol (-o-) and 17 alpha-estradiol (-•-) on the RCF-8 cell line after seven days in culture. All values (mean ± S.D., n= 9 to 30) for treatments with 17 beta-estradiol were significantly ($p < 0.05$) different than the control value. Values for treatments with 17 alpha-estradiol (n= 9 to 18) were not statistically different than control.

Figure 1B: Blockade of the 17 beta-estradiol effect (1pM) with tamoxifen (100pM) in RCF-8 cells. Treatment values are compared to the number of cells present at 7 days in culture in the absence of added hormone (n = 9 to 30).

FIGURE 1

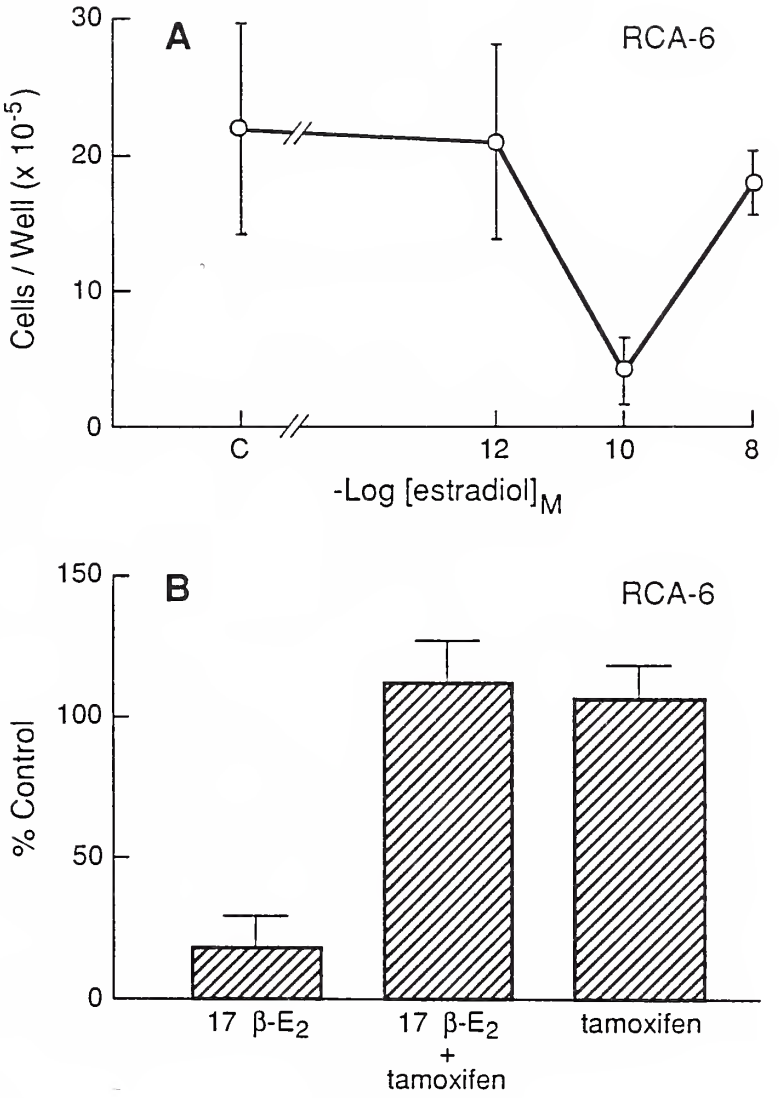

A
RCF-8
Cells / Well (x 10-3)
25
20
15
10
5
0
C
16
14
12
10
8
6
-Log [estradiol]M
B
RCF-8
% Control
400
300
200
100
0
17 β-E2
17 β-E2 + tamoxifen
tamoxifen

The effects of estradiol on another cell line which exhibited estradiol binding, RCA-6, were also examined. In contradistinction to RCF-8, this cell line continues to proliferate and increase in number when grown in serum-free conditions. After 7 days in vitro the initial 10^5 cells increased to $2.19 \pm 0.76 \times 10^6$ (mean $\pm$ SD, n=33). 17 beta-estradiol significantly ($p<0.05$) decreased the number of cells present on day 7 (Figure 2A). The maximal effect occurred with a concentration of 100 pM. At this dose, 17 alpha-estradiol had no effect on cell number. Tamoxifen (100 pM) completely blocked the inhibitory effect of estradiol, although it had no effect alone (Figure 2B). No changes in cellular morphology were evident in the RCA-6 cells after seven days of exposure to either 17 alpha- or 17 beta-estradiol.

Figure 2A: Effect of 17 beta-estradiol (-o-) on the RCA-6 cell line after seven days in culture. Treatment values (mean $\pm$ S.D., n = 9 to 33) obtained at 100pM and 10 nM 17 beta-estradiol were significantly ($p<0.05$) different from control.

Figure 2B: Blockade of the 17 beta-estradiol (100pM) effect with tamoxifen (100pM) in the RCA-6 cell line. Treatment values are compared to cells grown in the absence of hormone (n = 9 to 18).

FIGURE 2


A
RCA-6
Cells / Well (x 10^{-5})
30
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10
8
-Log [estradiol]$_M$
B
RCA-6
% Control
150
100
50
0
17 β-E$_2$
17 β-E$_2$ + tamoxifen
tamoxifen

The RCF-12 cell line was used as a control because it had no detectable estrogen binding. No significant differences in cell number compared to untreated cells were present after seven days of exposure to 17 beta-estradiol (10^{-12}M, 10^{-10}M and 10^{-8}M; data not shown).

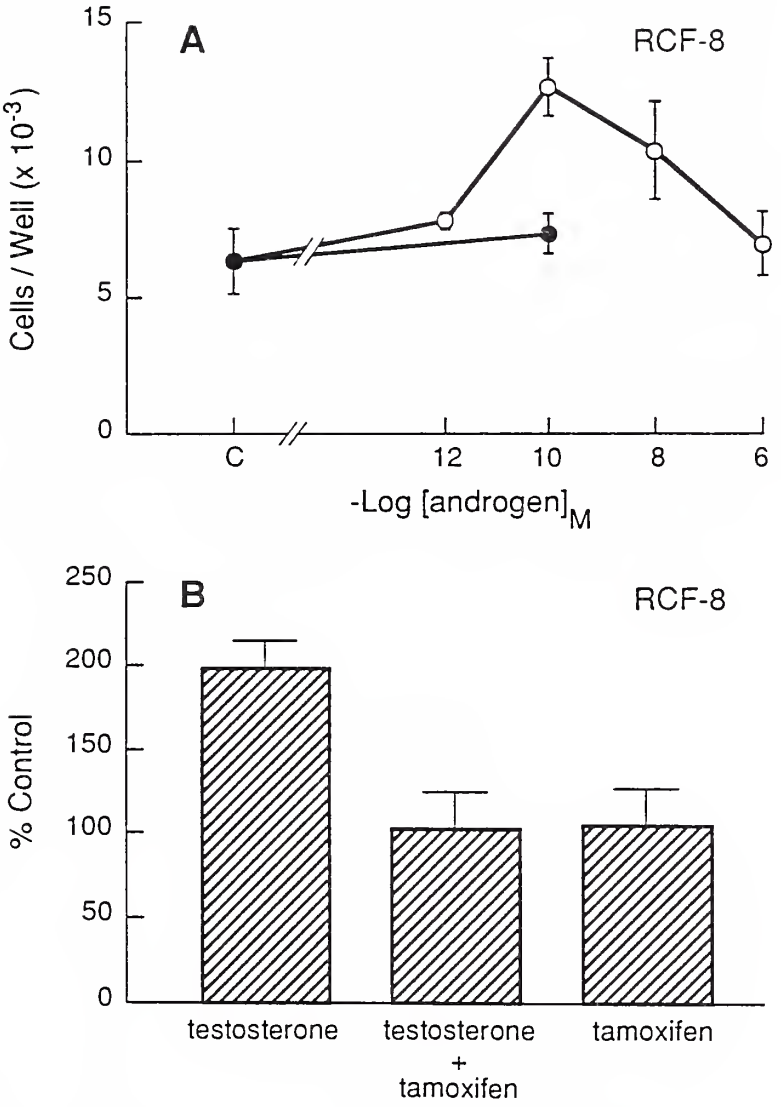
Effects Of Testosterone And Dihydrotestosterone On Cell Growth

The response of the RCF-8 cell line to testosterone, an aromatizable androgen, and dihydrotestosterone (DHT), a non-aromatizable androgen is shown in figure 3A. Testosterone, at 100pM and 10nM had a significant ($p<0.05$) positive effect on cell number. When used at the concentration that produced a maximal effect for testosterone, 100 pM, DHT had no significant effect on cell number. As with 17 beta-estradiol, the enhancement of survival by testosterone declined with concentrations above 100 pM. Tamoxifen (100 pM) completely blocked the maximal testosterone response in the RCF-8 cell line (Figure 3B).

Figure 3A: Effect of testosterone (-o-) and dihydrotestosterone (-•-) on the RCF-8 cell line after seven days in culture. The values obtained (mean ± S.D., n = 9 to 15) for treatment with testosterone at 100pM and 10nM were significantly ($p<0.05$) different than the control value.

Figure 3B: Blockade of the testosterone effect (100pM) with tamoxifen (100pM) in the RCF-8 cell line. Treatment values are compared to cells grown in the absence of added hormone (n= 9 to 15).

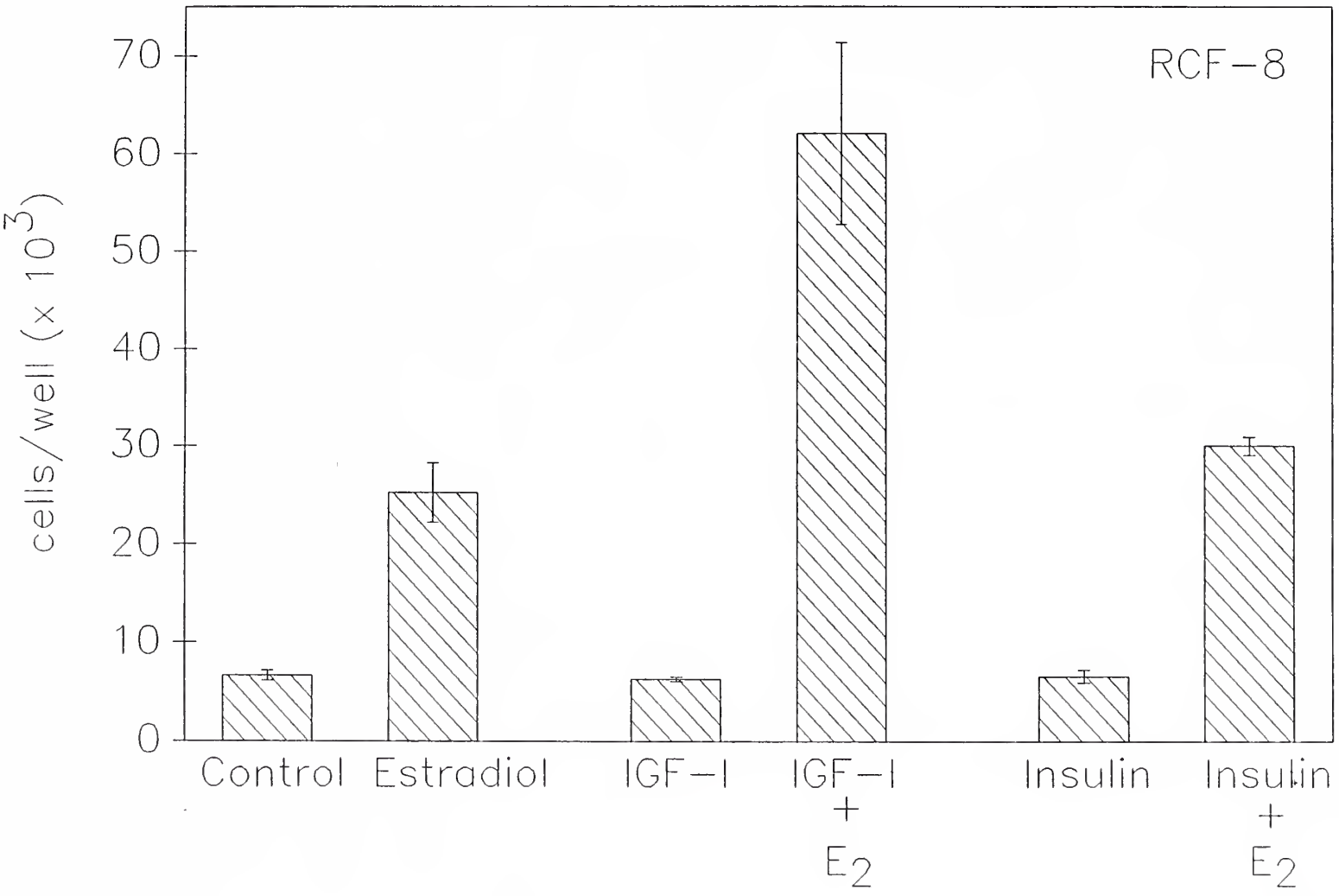
FIGURE 3


A
RCF-8
Cells / Well (x 10-3)
15
10
5
0
C
12
10
8
6
-Log [androgen]M
B
RCF-8
% Control
250
200
150
100
50
0
testosterone
testosterone + tamoxifen
tamoxifen

Interaction between Estradiol and IGF-I on cell growth

Figure 4 shows that co-addition of IGF-I (0.1nM) and estradiol (1pM) results in a synergistic increase in proliferation of the RCF-8 cells. While estradiol alone is able to stimulate the in vitro survival of RCF-8 cells, the RCF-8 cells need the presence of estradiol to be stimulated by IGF-I. On the other hand, a dose of 1nM insulin which has been shown to be optimal for stimulating cell growth in other hypothalamic cell lines (65), did not produce any of the same effects as did IGF-I. Insulin alone (100nM) or in combination with estradiol (1pM) has no effect on cell growth (figure 4).

Figure 4: Effect of estradiol, IGF-I and insulin on the RCF-8 cell line after seven days in culture. The value obtained (mean $\pm$ S.D., n= 9-21) for treatment with estradiol (1pM) and IGF-I (0.1nM) was significantly ($p<0.05$) different than the value for treatment with estradiol (1pM) alone. The value obtained for treatment with estradiol (1pM) and insulin (100nM) was not significantly different than the value obtained for treatment with estradiol (1pM) alone.


RCF-8
cells/well (x 10^3)
70
60
50
40
30
20
10
0
Control
Estradiol
IGF-I
IGF-I + E2
Insulin
Insulin + E2

In other studies, another cell line, RCF-12, was found to respond to IGF-I in an estradiol-independent manner. IGF-I alone induces a marked proliferative response (results not shown).

DISCUSSION

These studies demonstrate the potential of 17 beta-estradiol (E_2) and testosterone (T) to alter the growth patterns of cells derived from the fetal rat hypothalamus. These clonal cell lines were established to enable us to investigate the effects of individual hormones on the growth and development of homogenous populations of developing hypothalamic cells. The RCF-8 and RCA-6 cell lines were chosen for their high E_2 binding capacity, when compared to thirteen other similar cell lines.

We employed serum-free conditions in order to create a model in which to test for possible neurotrophic effects of specific sex steroids. These conditions in which the number of hypothalamic cells is progressively decreasing are very similar to those used to test potential neurotrophic factors in other cell types (55-58) and they mimic, in part, the normal programmed cell death which occurs in the perinatal rat brain (53-54).

The survival of RCF-8 cells was increased by E_2. This effect was greatest at the physiologic concentration of 1 pM. The lack of effect of 17 alpha-estradiol and the positive effect of higher concentrations of estriol strongly support the hypothesis that this effect is mediated via specific estrogen receptors. The role of

estrogen receptors is further confirmed by the complete blockade of the effect with tamoxifen.

The apparent attenuation of the survival effect in response to E_2 concentrations above 1 pM remains unexplained. Decreasing effectiveness of higher concentrations of estradiol on cell growth has been reported previously (41). This effect could result from desensitization of the estrogen receptors, or from the estrogen-induced expression of other genes which might limit the survival effects. It is also possible that estrogen could be inducing differentiation of the cells, which is often associated with decreased proliferation. Estrogens have been documented to increase the expression of the cellular proto-oncogenes, c-fos and c-myc (71) and to induce the production of autocrine and paracrine growth factors (72-73) which could promote differentiation.

The androgens, T and DHT were examined to further explore the possibility that RCF-8 cells might serve as a model of the cellular events involved in generation of sexual dimorphisms in the hypothalamus. Numerous studies have demonstrated that sexual differentiation of the rodent hypothalamus is dependent on the presence of either estrogens or aromatizable androgens, and can be blocked by antiestrogens (8). A similar specificity was observed in the present studies with respect to steroid-induced survival of RCF-8 cells. The maximal T effect was seen at

concentrations two log orders of magnitude higher than those required for E_2. The inability of equimolar concentrations of DHT to reproduce the T effect strongly suggests that aromatization of T mediates this response. In preliminary direct enzymatic studies done in collaboration with Dr. Naftolin's laboratory, the RCF-8 cell line has been demonstrated to contain aromatase activity (74).

We conclude that these cells exhibit the critical elements involved in sexual differentiation of the developing hypothalamus: i.e. they are estradiol-binding hypothalamic-derived cells that exhibit a response to E_2 or T, but not to the non-aromatizable androgen DHT. Furthermore, the presence of aromatase indicates that each of the biochemical mechanisms required for response to aromatizable androgens may coexist within the same cell. Previous studies on the occupation of estrogen receptors in the brain after exposure to aromatizable androgens have suggested a close functional and structural relationship between the aromatase enzyme and the estrogen receptor system, since low levels of estrogen biosynthesis in the brain are sufficient to occupy a substantial proportion of the available estrogen receptor binding capacity (75-76). However, the question of whether aromatase is present within the estrogen target neurons themselves, or in closely neighboring cells, has remained unanswered. Although SV-40 viral transformation could clearly have

altered the properties of the cells with respect to their content of steroid receptors and steroid metabolizing enzymes, it seems possible that the normal hypothalamic cell from which the RCF-8 line was derived may have represented an estrogen-responsive, aromatase-containing neuron.

In contrast to the results with the RCF-8 cells, estradiol decreased the proliferation of RCA-6 cells. Compared to RCF-8, this cell line has a much shorter doubling time in serum, has one tenth the estradiol binding capacity, and proliferates in the absence of serum. The ability of E_2 to decrease the rate of proliferation of these cells also appeared to be estrogen receptor mediated as demonstrated by the effects of 17 alpha-estradiol and tamoxifen. Estrogens have been documented to decrease the growth rate of other cell types via specific estrogen receptor mediated events (see 46 for review). In addition, Soto and Sonnenschein (46) have suggested that estrogens may induce cell multiplication inhibitors. Although this inhibition of proliferation could indicate that E_2 is inducing differentiation, no phenotypic change in RCA-6 cellular morphology was noted after exposure to estradiol.

These studies demonstrate the ability of estradiol to modulate the growth patterns of transformed hypothalamic cell lines via specific estrogen receptors. These effects were present at physiologic concentrations and could be

reproduced by the aromatizable androgen testosterone, but not by the non-aromatizable androgen DHT. In the RCF-8 cells deprived of serum, a system in which cell number is progressively decreasing, E_2 exhibited neurotrophic activity. In the RCA-6 cells which proliferate even in the absence of serum, E_2 decreased the growth rate.

In addition to establishing cell lines which can be used to study sexual dimorphism, we were interested in using the model cell line to examine the possibility that estradiol exerts it´s effects by modulation of response to other local growth factors. For this purpose we selected IGF-I and insulin which are known to be present at high concentrations in fetal brain. Our results indicated that estradiol can modulate the growth-promoting effects of IGF-I on a hypothalamic cell line which express nuclear estrogen receptors. The cell line without estrogen receptors (RCF-12) neither responded to estradiol nor altered its response to IGF-I by co-exposure to estradiol.

RCF-8 cells are not responsive to IGF-I in the absence of estradiol. One possible explanation is that estradiol induces the appearance of type I IGF-I receptors in RCF-8 cells. However, we have been unable to detect any differences in the amount or affinity of IGF-I binding sites in RCF-8 cells grown either with or without estradiol (77). Thus, estradiol may be necessary to activate an as of yet undetermined intracellular mitogenic pathway normally

triggered by activated IGF-I receptors. In accordance with this possibility estradiol has recently been proposed to modulate the coupling of striatal and pituitary dopamine receptors to the adenylate cyclase system normally involved in postreceptor activation mechanisms (78). In addition, estradiol treatment has no effect on the growth of the cells treated with insulin. We utilized insulin in these studies as a control to monitor the specificity of the IGF-I/estradiol synergy. Insulin is a peptide growth factor which is structurally closely related to IGF-I suggesting that a similar synergy between insulin and estradiol could be expected. It is present in high levels in both the developing brain and the adult brain (79). The concentration of insulin employed, 100nM, is a concentration known to saturate the insulin receptor in other hypothalamic cell systems (80). The selectivity of the estradiol effect on IGF-I responsiveness but not on insulin responsiveness suggests that the IGF-I/estradiol interaction may play a unique and significant role in the development of the fetal brain, although the exact effects and mechanisms remain to be determined.

Other authors have suggested that a possible mechanism whereby ubiquitous growth factors (such as the IGFs or fibroblast growth factors) may specifically affect particular subsets of cell populations during brain development is by interactions between these growth factors

and other molecules present in the cellular environment, each working at a different phase of the cell cycle (81-83). Our data suggests that one such mechanism might be explained by the ability of estradiol to allow some estrogen sensitive cells to respond to IGF-I.

We conclude that sex steroids have considerable potential to directly and indirectly alter the survival or growth characteristics of developing hypothalamic cell lines and that these cell lines give us a unique chance to study the developmental process of sexual dimorphism using an in vitro system. In addition, this work suggests that the presence of estrogen at particular stages during neurogenesis may constitute a regulatory mechanism whereby some estrogen-sensitive cells will start to respond to mitogenic/survival signals, such as IGF-I, already present in their microenviroment.

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