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An Assessment of the Agonist/Antagonist Effects of Tamoxifen on the Activation of Sexual Receptivity and the Induction of Hypothalamic Progestin Receptors in the Female Mouse

by

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ABSTRACT

Antiestrogens (AE) are powerful biological tools used to elucidate the molecular mechanisms of estrogen (E) action. Tamoxifen (TAM) is a well known AE that is the treatment of choice for E receptor positive mammary cancer. Despite its reputation as an antagonist, estrogenic effects of TAM, such as the induction of uterine growth in female mice and the potentiation of aggression in intact male mice also have been noted. The present study examined the effect of TAM on two estrogen regulated processes, the activation of lordotic behavior and the induction of progestin receptors (PR) in the hypothalamic-preoptic area (HPOA) in female mice. The effects of TAM, in the dosage range 0.5 - 100 μg, were studied with and without the presence of 5 μg estradiol benzoate (EB) to address the possibility of agonist and antagonist effects. None of the TAM treatments activated lordosis or induced PR in the HPOA. TAM antagonized EB-activated lordosis in a dose dependent fashion, but did not totally suppress lordosis even at the highest dose. However, all doses of TAM suppressed EB-induced PR levels to those seen in oil-treated mice. Differences noted in the manner in which rats responded to TAM for the same responses indicated a species-specific response to TAM. Interestingly, variability in the action of TAM exists in mice as indicated by its ability to potentiate aggression in males yet block lordosis and PR induction in females. Due

to the sexually dimorphic nature of the neural substrates for these responses, it was postulated that the early hormonal environment may organize the eventual response to TAM. One possible mechanism is the hormonal regulation of chromatin organization which governs the availability of DNA sequences to which the receptor must bind to regulate gene transcription. Another possibility explored is the variable action of transcriptional activating factors located on the estrogen receptor and the degree of synergism with species-, tissue- and cell-specific transcription factors.

CHAPTER ONE

INTRODUCTION

In broad terms, the aim of behavioral endocrinology is to explain hormonal influences on behavior. To this end, hormonally regulated behavior patterns that can be reliably elicited and quantified serve as models. Hormonal conditions are manipulated to determine the effects on the behavior in question. One of the most thoroughly studied sexual responses is lordosis, a reflex exhibited by a sexually receptive female rodent upon being mounted by a male. This response is characterized by a concave arching of the back, elevating both the head and the rump, exposing the genitalia (see Figure 1), and it is recognized as a primary indicator of sexual receptivity.

Hormonal Regulation of Sexual Receptivity

Copulation in female rodents is dependent upon the lordosis response; unless the female displays this posture, intromission and ejaculation by the male are not possible (Diakow, 1974; Pfaff, Diakow, Montgomery, & Jenkins, 1978). The display of lordosis is dependent upon ovarian hormones estrogen (E) and progesterone (P). Ovariectomized rodents, whose endogenous source of E and P was removed, do not exhibit the lordosis response (Boiling & Blandau, 1939). Upon replacing E and P in physiological doses, the lordosis response can be restored (Edwards, Whalen, & Nadler 1968; Whalen, 1974). This method of removing the endogenous

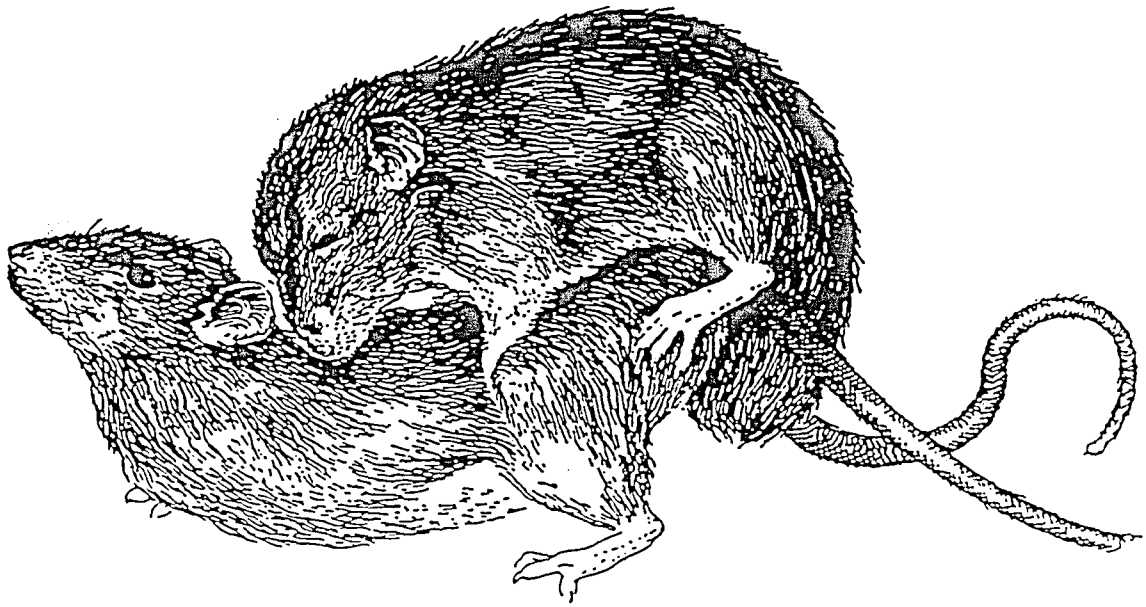

Figure 1. Lordosis posture of the rat. Reprinted from S. A. Barnett, The Rat: A study in Behavior, Chicago, 1963.

source of hormones by castration and replacing the hormones exogenously became known as the castration-replacement paradigm. It was soon discovered that lordosis could be brought about in ovariectomized rats (Davidson, Rodgers, Smith, & Bloch, 1968; Edwards et al., 1968), hamsters (Carter, Michael, & Morris, 1976), guinea pigs (Young, 1969) and mice (Ring, 1944) solely by high doses of E (100μg). This discovery led to the distinction between the priming and the activational effects of E. When a physiological dose of E is administered 24-48 h prior to P administration and lordosis results, we speak of the ability of E to prime the neural substrate for sexual receptivity. Without prior administration of E, P cannot bring about sexual receptivity. However, when higher doses of E (100 μg) are administered without P, the lordosis response can be elicited. In this case we speak of the activational effects of E. This is an important distinction that is necessary for the elucidation of the neural mechanisms regulating sexual behavior because in vivo, this behavior is regulated by both E and P. Therefore, to understand the induction of sexual receptivity, the hormonal conditions utilized must reflect those that naturally occur.

Neural Substrates that Mediate Sexual Receptivity

A great deal of research has sought to determine the

brain sites mediating the effects of E and P on sexual receptivity. Autoradiographic studies, in which tritiated hormones such as E are injected systemically, enabled the identification of brain regions that selectively retained E. A number of E-concentrating sites in the hypothalamus, medial preoptic area, amygdala, and pituitary of both mice and rats were identified (Pfaff, 1968; Pfaff & Keiner, 1973; Stumpf & Sar, 1975). Implant studies, which are a refined version of the castration-replacement paradigm in which minute amounts of crystalline steroids are directed to particular brain regions, enabled experimenters to pinpoint the location of behaviorally active cell groups. This led to the identification of the ventromedial hypothalamus (VMH) as the most effective site for E implants to activate sexual receptivity in rats in the absence of P administration (Barfield & Chen, 1977; Dörner, 1968; Lisk, 1962). Recall, however, that in vivo both E and P regulate sexual behavior. In order to mimic the natural circumstances in which E primes the neural substrate for lordosis dilute implants of E were utilized in other studies. When dilute implants of E were directed to the ventromedial nucleus of the hypothalamus (VMN), priming, but not activation of sexual receptivity resulted, while implants in other areas such as the preoptic area (POA) were virtually without effect (Davis, McEwen, & Pfaff, 1979; Rubin & Barfield, 1980). A combination of the implant and autoradiographic techniques

enabled experimenters to determine that the degree of diffusion of hormones from the implant sites was minimal (within 0.5mm), thus more firmly establishing these sites as behaviorally effective (Davis, Krieger, Barfield, McEwen, & Pfaff, 1982). Additional evidence for the role of the VMN in lordosis came from lesion studies in which the VMN or its efferent (outgoing) pathways were destroyed, resulting in abolished or severely disrupted sexual responsiveness (Malsbury, Strull, & Daood, 1978; Matthews, & Edwards, 1977). In addition, anisomycin, a protein synthesis inhibitor, and tamoxifen, an antiestrogen, blocked the facilitation of E and P activated lordosis when implanted into the VMN, but not in the POA or interpeduncular region (Glaser & Barfield, 1984; Rainbow, McGinnis, Davis, & McEwen, 1982).

Utilizing similar experimental methods the site of action of P also was examined. Autoradiography indicated that P-concentrating sites were localized to the hypothalamus, preoptic area, amygdala, midbrain, and cortex in a number of species (Blaustein & Feder, 1979; Kato & Onuchi, 1977; Moguilewsky & Raynaud, 1977; Warembourg, 1978). Utilizing doses of E sufficient to prime but not activate lordosis, the effect of P implants in a number of locations was examined. Only implants in or immediately adjacent to the VMN were behaviorally effective (Rubin, & Barfield 1983a,b). This is interesting in view of several

reports that P implants in the midbrain stimulated lordosis in E-primed rats (Luttge & Hughes, 1976; Ross, Claybaugh, Clemens, Gorski, 1971; Yansae & Gorski, 1976). However, the midbrain and the hypothalamus respond differently to E priming. Estradiol treatments increased the concentration of progestin receptors (PR) in the hypothalamus but not the amygdala, cortex or midbrain of rats and guinea pigs (Blaustein & Feder, 1979; MacLusky & McEwen, 1978, 1980; Moguilewsky & Raynaud, 1979). While PR induction is not sufficient for the activation of sexual receptivity, it has been correlated with the appearance of this behavior (further treatment of this issue is beyond the scope of the present discussion; for reviews see Barfield, Glaser, Rubin & Etgen, 1984; Etgen, 1984).

Hormone Receptors: Mechanism of Action and Binding Characteristics

Another area of research which has received considerable attention in the past two decades is the biochemical mechanism of hormone action. The currently accepted mechanism of hormone action is depicted in Figure 2. Hormones such as E and P diffuse across the cell membrane where they bind to a receptor located in the nuclear compartment (King & Green, 1984; Welshons & Gorski, 1988). Receptors possess the following binding characteristics: (i) steroid specificity, only hormones of the same class compete effectively for binding their

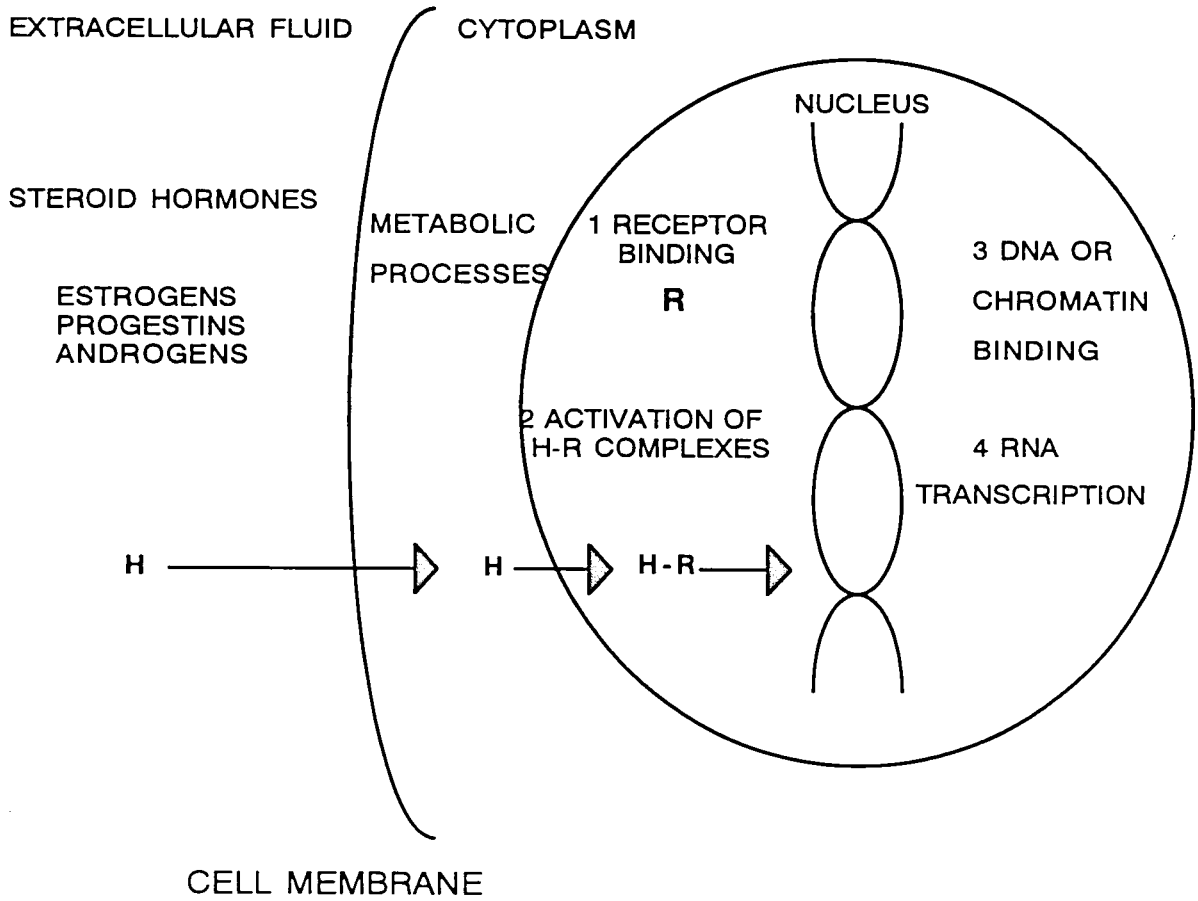

Figure 2. Mechanism of hormone action.

receptor. For example, androgens, will not compete with radiolabeled estrogen ([^{3}H]E) for E receptor (ER) binding, whereas diethylstilbestrol (DES), a synthetic estrogen will compete with [^{3}H]E for ER; (ii) tissue-specificity, receptors are present in significantly greater amounts in target tissues compared to nontarget tissues; (iii) high affinity, because blood levels of hormones are usually 10^{-10} - 10^{-8} M, if the tissue is to respond to the hormone via a receptor mechanism, the receptor must have an affinity for the hormone which is in the range of the blood levels; (iv) saturability, because the biological response to a hormone is saturable, there must be a finite number of binding sites for the hormone; (v) correlation with biological response, it must be demonstrated that binding of the hormone by its receptor results in a biological response (for review see Clark & Peck, 1977). Upon binding hormone, the receptor undergoes a ligand-induced conformational change termed activation, which enables the receptor to bind with high affinity to nuclear acceptor sites on specific genes and alter ribonucleic acid (RNA) and protein synthesis (Rories & Spelsberg, 1989; Thrall, Webster, & Spelsberg, 1978).

These findings on biochemical mechanisms were important for behavioral research because it is this alteration in gene expression that is thought to underlie the hormonal regulation of behaviors such as lordosis. Evidence that the

activation of lordosis requires protein synthetic steps comes from studies that utilized protein synthesis inhibitors, such as anisomycin. When anisomycin was administered systemically (Rainbow, Davis, & McEwen, 1980) or implanted into the VMN (Glaser & Barfield, 1984; Rainbow, McGinnis, Davis, & McEwen, 1982) it blocked the facilitation of lordosis by E and P in ovariectomized rats.

In addition to protein synthesis inhibitors, other chemicals have been utilized to determine the role of E and P in the regulation of sexual receptivity. Among these are E agonists and antagonists. An E agonist is a substance that mimics the effects of E; it works in a manner similar to E to control gene regulation. An E antagonist or an antiestrogen (AE) is a substance capable of blocking the effects of estrogen.

Action of Tamoxifen in Peripheral and Neural Tissue

Antiestrogens are powerful biological tools that can be used to elucidate the mechanism of estrogen action at the molecular level. Tamoxifen (2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N.dimethylethanamine -- TAM), is a synthetic AE that is used primarily for the treatment of estrogen receptor-positive breast cancer. TAM inhibits the proliferation of E receptor-containing breast cancer cells, as well as E-stimulated protein synthetic activities including the induction of PR (Freiss, Prebois, Rocheforte,

& Vignon, 1990; Katzenellenbogen, Miller, Mullick, & Sheen, 1985). Competition for the estrogen receptor is widely accepted as the mode of antagonistic action of TAM (Horwitz & McGuire, 1978; Katzenellenbogen, Bhakoo, Ferguson, Lan, Tatee, Tsai, & Katzenellenbogen, 1979). This is evident since the sensitivity of different breast cancer cell lines to the growth suppression effects of TAM is correlated with their ER content. The growth of MCF-7 breast cancer cells, which possesses a high concentration of ER, is inhibited markedly by TAM, whereas the growth of T47D cells, which possess relatively fewer ER, is inhibited only minimally (Katzenellenbogen, et al., 1985).

Similar to other antiestrogens, TAM has been classified as a mixed agonist-antagonist due to its ability to facilitate, as well as block, estrogen-dependent responses in a number of cell types and a variety of species (see Pasqualini, Sumida, & Giambiagi, 1988 for review). When administered to ovariectomized rats in combination with estradiol benzoate (EB) TAM antagonizes EB-induced progesterone receptors (PR) in the uterus (Castellano-Diaz, Gonzalez-Quijano, Liminana & Diaz-Chico, 1989; Kirchhoff, GrÜnke, Hoffmann, Nagel, Ghraf, 1983), the pituitary (Kirchhoff et al, 1983) and the hypothalamic preoptic area (HPOA) (Etgen & Shamamian, 1986). Antagonism in rats has also been reported for the activation of lordotic behavior (Etgen & Shamamian, 1986), and the induction of uterine

growth (Harper & Walpole, 1967). In the absence of estrogenic stimulation, however, partial agonism is evident in the increased concentration of PR in the uterus (Castellano-Diaz et al., 1989; Kirchhoff et al., 1983), pituitary (Kirchhoff et al, 1983) and HPOA (Etgen & Shamamian, 1986) as well as increased uterine weight (Harper & Walpole, 1967). In addition, TAM is estrogenic in its ability to induce synthesis of uterine complement component C3, produced in the luminal and glandular epithelial cells of the rat uterus (Sundstrom, Komm, Xu, Boundy, Lyttle, 1990).

Variability of the effects of TAM is evident when one reviews research utilizing the guinea pig. In both the fetal and the neonatal guinea pig, TAM is as uterotrophic as E. Further, when administered with E present, TAM does not block but potentiates estrogen's effects (Pasqualini, Sumida, Giambiagi, & Nguyen, 1987). However, TAM is not purely an agonist for the guinea pig uterus. TAM alone has no stimulatory effect on histone acetylation, whereas estrogen stimulates acetylation 10-fold. When TAM is co-administered with E, antagonism of histone acetylation results (Pasqualini, Cosquer-Clavreul, & Gelly, 1983). For PR induction, TAM is only a partial E agonist in the fetal uterus of the guinea pig, but a full agonist in the neonatal uterus (Pasqualini et al., 1987). As in the uterus, TAM is as trophic as E in both the fetal and neonatal vagina, but

unlike the uterus regarding the induction of PR, TAM is only a partial agonist for both fetal and neonatal guinea pigs (Nguyen, Giambiagi, Mayrand, Lecerf, & Pasqualini, 1986).

The literature regarding the effects of TAM in mice is both sparse and inconsistent depending on the response under consideration. The controversy over its antagonist effects in mice began in 1971 when Terenius reported that TAM was as uterotrophic as estrogen in the mouse and that it did not block estrogen-induced uterine growth. There has been a recent report of the antiuterotrophic effects of TAM in the dosage range of 0.001 - 100 μg (Pavlik, van Nagell, Nelson, Gallion, Donaldson, Kenady, & Barankowska-Kortylewicz, 1986). In this dosage range, TAM + EB (0.05 μg) treated mice exhibited only slight suppression of uterine weight, 220 - 300% of the noninjected controls compared to EB-treated mice whose uterine weight was 300% of the noninjected control. PR induction of the TAM + EB-treated mice was 350 - 450% of noninjected control versus 450% noninjected control for EB-treated mice. An alternate interpretation might classify this as a partial agonist effect.

Another area where agonist properties of TAM have been reported is the potentiation of estrogen-activated aggression in intact male mice (Simon & Perry, 1988). Intact TAM-treated males (dosage range of 50.0 - 400.0 μg

TAM) had a significantly greater number of attacks against stimulus males compared to oil-treated males. There were no significant dose effects. Due to aromatization of testosterone to E, intact males have an endogenous source of E. One would expect TAM to decrease aggressive behavior based on its action as an antiestrogen, through competition for ER binding. However, TAM potentiated aggression above the level seen in oil-treated intact males, thus apparently acting as an E agonist.

It therefore seems reasonable to conclude that the effect of TAM seems to be a function of the species, target tissue and response under consideration, and that further experimentation is needed to provide additional information on the action of TAM. In this study, the effect of TAM on two estrogen-regulated processes, the activation of lordotic behavior and the induction of PR in the HPOA were examined using CFW female mice as a model. The effects of TAM on these processes were studied with and without the presence of EB to address the possibility of agonist and antagonist effects. Although a direct examination of the mechanism of action of TAM was beyond the scope of these studies, it was hoped that the data obtained would aid in developing hypotheses concerning the molecular events that mediate the agonist/antagonist actions of TAM in the CNS of the female mouse.

CHAPTER TWO

METHODS

Animals

Sexually inexperienced female CFW mice (70-75 days of age) purchased from the Charles River Breeding Farm (Wilmington, MA) were housed in groups of four in 28 X 28 X 13-cm polycarbonate cages lined with wood chips until treatments were initiated. They were maintained on a 12:12 hr reversed light/dark cycle with lights on at 1900 hr with food and water available at all times. Mice were bilaterally ovariectomized two weeks prior to use. They were anesthetized with an intraperitoneal injection of Nembutal supplemented with the inhalation anesthetic Metofane. Surgery involved a 2 mm incision along the lateral sides of the spinal cord posterior to the last rib. After the ovaries were located and removed the peritoneum was sutured shut and the incision closed with a wound clip. All maintenance procedures were in compliance with Federal guidelines for animal care. Ten mice were assigned to each treatment group.

Treatments

Estradiol benzoate (EB) and progesterone (P) were purchased from Steraloids (Wilton, NH), and TAM was purchased from Sigma (St. Louis, MO). In Experiment One, the females were randomly divided among the following treatments: (i)TAM: either 0.5, 2.5, 5.0, 10.0 or 100.0 μg;

(ii) EB: 5.0 μg; (iii) oil: vehicle only. Forty-two hours after the administration of these treatments, each female received a 500 μg P injection. In Experiment Two, females were given a combined treatment consisting of 5.0 μg EB with either 5.0, 10.0, or 100.0 μg TAM, followed by 500 μg P as above. All treatments were administered subcutaneously in 0.02 cc oil vehicle between 1500-1600 hr (8-9 hr into the dark cycle) and continued once a week for 4 weeks.

Behavioral studies

Measurement of lordotic behavior was conducted 6 hours after P administration between 1500-1900 hr, 8-12 hr into the dark cycle under dim red illumination. Each test consisted of placing females individually into a clear polyethylene cage, 51 x 41 x 22 cm, lined with wood chips, 5 minutes prior to introduction of a stud male mouse. The stud males were given access to fully receptive females prior to sessions with the experimental females. All females were tested until they received 10 mounts or 30 minutes of exposure to the stud male. A lordosis quotient (LQ), calculated as the percentage of mounts resulting in a lordosis response, was used a measure of sexual receptivity. Tests were conducted once a week for 4 weeks.

Progestin Receptor Assay

The week following the final behavioral test mice from each group received TAM, EB, TAM+EB or Oil injections 42 h prior to sacrifice by cervical dislocation. Their brains

removed rapidly and blocked on ice. The HPOA was removed as a single block. The section was bordered posteriorly by the mammillary bodies, laterally by the hypothalamic sulci, and anteriorly by a cut approximately 2 mm anterior to the optic chiasm to a depth of approximately 2mm.

Subsequent steps were performed at 0-4°C. Tissue was homogenized in 500 μl of fresh ice-cold TEDGM buffer (10mM Tris HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 20% glycerol (v/v), 10mM sodium molybdate, pH=7.4 at 0° C) by 20 strokes in a glass-teflon homogenizer followed by a 500 μl wash and 8 additional strokes. The homogenate and wash were combined and centrifuged for 10 minutes at 2000 x g in a fixed angle rotor in an IEC Centra 7R centrifuge. The supernatant was centrifuged for 60 min at 100,000 x g using an SW50.1 rotor in a Beckman L8-70 Ultracentrifuge. The supernatant was immediately used in the assay.

Incubations were conducted in 12 X 75 mm glass tubes that were pretreated with 0.1% BSA prior to use. The incubate consisted of 200 μl of cytosol, 40 μl of 0.4 nM [^{3}H]R5020 (New England Nuclear, Boston MA; S.A. 84.7 Ci/mmol) and 10 μl of buffer without (for measuring total binding) or with (to assess non-specific binding) 100x excess of unlabeled R5020. Samples were thoroughly mixed and incubated at 0° C for 8 hr.

The incubation was terminated by the addition of 250 μl of hydroxylapatite (HAP) suspension (50% (v/v) HAP, 50%

(v/v) TE buffer; pH=7.4 at 0°C) into the incubation tubes. The tubes were immediately vortexed at moderate speed for 10 seconds and this step was repeated every 10 minutes for 30 minutes. The incubates were then centrifuged for 4 minutes at 1000 x g in a swinging bucket rotor. The supernatant was aspirated, and the HAP pellet washed 4X in 2 ml of TE containing 1% Tween 80 (polyoxyethylenesorbitan monooelate; pH=7.4). Each wash was followed by a 3 minute centrifugation at 1000 X g.

After the final wash the walls of the tubes were cleaned with ethanol and dried with cotton swabs. The washed pellets were extracted in 1 ml of ethanol with periodic vortexing and then centrifuged for 3 minutes at 1000 x g. A 900 μl aliquot was taken and placed in a 20 ml scintillation vial. The volume of ethanol was brought back to 1 ml and the procedure was repeated again. Ten ml of toluene based scintillation fluor was added and the samples were counted in a Beckman LS-8100 liquid scintillation counter. Efficency was determined by the external standard channels ratio method.

Specifically bound hormone was calculated by subtracting nonspecific from total binding. Protein content was measured using the Bio-Rad Dye-Binding Reagent Kit and all data were normalized by converting to a per mg protein basis. Protein values for the incubates ranged from 0.37 - 0.45 mg.

Data Analyses

The sexual behavior of the female mice was highly variable in the EB + P group over the first two weeks of testing and was stable over weeks 3 and 4. On this basis, analyses of the behavioral data were based only on the last two tests for all groups. In addition, data from females that were mounted less than 10 times during the course of a behavioral test were excluded from the analysis for that test to ensure that all females that had an equal opportunity to express the behavior under study. Biochemical data were analyzed by examining the relative induction of PR in the experimental groups in comparison to that seen in females that received EB + P and then EB alone the week of the assay.

CHAPTER THREE

RESULTS

Experiment 1: Does Tamoxifen Act As An Estrogen Agonist?

Figure 3A shows the mean LQ's for each of the groups. It is evident from this figure that only the EB + P treatment led to the display of sexual receptivity. Mice in this condition had a mean LQ of 59.7. None of the TAM + P or Oil + P treated mice were sexually responsive; the mean LQ's for these groups ranged from 0 to 4.5.

Figure 4A shows the effect of the treatments on the induction of PR in the HPOA. Data from the experimental groups are presented as the percentage PR concentration measured in EB-treated mice which was 371.17 DPM/mg. Only the EB + P -treated mice exhibited significant induction of PR. The induction of PR in TAM-treated mice ranged from 3.2% to 7.0% of the EB-treated controls. These concentrations were similar to the basal levels of PR seen in the oil-treated mice (7.4% of EB-treated mice). The biochemical data is consistent with the behavioral data; only EB + P treated mice showed significant PR induction and were sexually responsive, whereas none of the TAM + P treatments led to the induction of PR or the activation of lordosis.

Experiment 2: Does Tamoxifen Act As An Estrogen Antagonist?

Figure 3B shows the mean LQ for TAM/EB + P treated mice. An unweighted means ANOVA for linear trend revealed a significant effect of TAM dosage ($F(1,23) = 4.88$, $p < 0.05$). The 5.0 μg TAM dose did not suppress the ability of EB + P to induce lordosis. The mean LQ for this group, 56.9, is similar to that of the mice that received only EB + P (59.7). However, as the dose of TAM was increased to 10.0 μg and 100.0 μg, mean LQs were suppressed to 41 and 20, respectively.

Figure 4B depicts the effect of TAM on the induction of PR by EB. All three TAM treatments suppressed [^{3}H]R5020 binding in comparison to that seen in the EB + P group, and there were no dose-related differences in this effect. EB/TAM treated mice exhibited only basal levels of PR induction (5.1 - 6.5% EB-treated mice) similar to the oil treated mice (7.4% EB-treated mice).

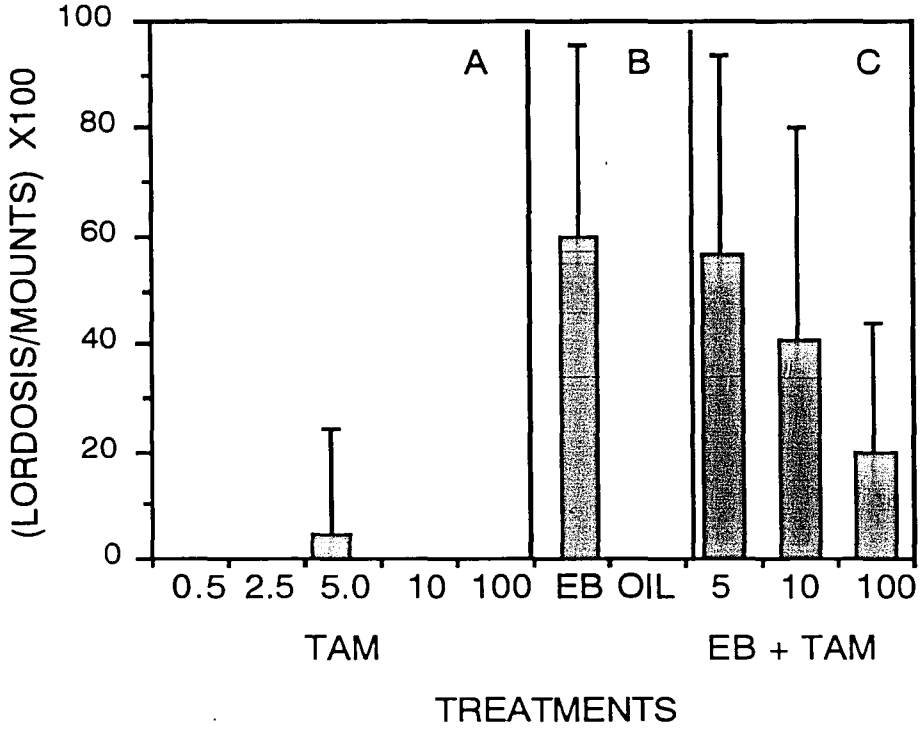

Figure 3.Effects of TAM alone (panel A) or TAM administered concurrently with simultaneous 5 μg EB (panel C) on lordosis. EB- and Oil- treated mice (panel B) served as positive and negative controls, respectively.

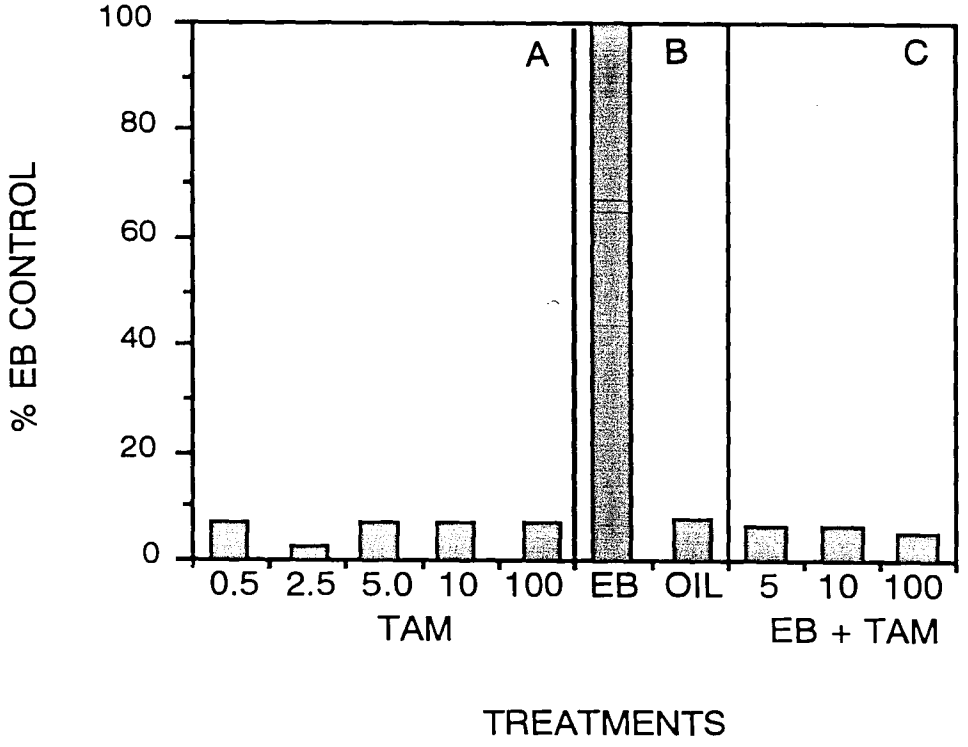

Figure 4. Effects of TAM alone (panel A) or TAM administered concurrently with simultaneous 5 μg EB (panel C) on HPOA progestin receptor content. EB- and Oil- treated mice (panel B) served as positive and negative controls, respectively. Data are expressed relative to EB-treated controls.

CHAPTER FOUR

CONSIDERATION OF RESULTS

The results from the first experiment indicate that TAM, in the dosages utilized in this study, did not act as a CNS agonist for the activation of lordotic behavior and the induction of PR in female mice. The latter finding differs from that reported with rats (Etgen & Shamammian, 1986), where a dissociation between the activation of lordosis and the induction of PR occurred. More specifically, rats administered 2.0 mg TAM 48 or 72 hr prior to sacrifice exhibited approximately a 150% and 200% increase in PR concentration respectively, in comparison to noninjected controls. However, neither of these groups were sexually responsive. This lack of sexual receptivity despite high levels of PR induction has been reported elsewhere for rats (Parsons, MacLusky, Krieger, McEwen, & Pfaff, 1979).

In the second experiment TAM blocked EB+P-activated lordosis in a dose dependent fashion. This is in accord with data obtained utilizing rats both when TAM was administered peripherally, or intracranially (Etgen, 1979; Howard, Etgen, & Barfield, 1984). Although no suppression occurred with equimolar doses of TAM and EB (LQ = 56.9), as the dose of TAM increased the degree of suppression increased. Interestingly, the biochemical response was more sensitive to the antagonist properties of TAM than the behavioral response; all TAM/EB treatments suppressed PR

induction to levels similar to oil-treated mice, while none of these treatments were able to totally suppress lordosis. The dissociation occurred most notably at the 5 μg TAM dose where behavior was unaffected despite the total suppression of EB-induced PR. Similarly, at the 10.0 and 100.0 μg TAM doses behavior was still evident, though suppressed, despite the lack of EB-induced PR. A similar dissociation of behavioral and biochemical effects of TAM has been reported for rats (Etgen, 1984; Etgen & Shammamian, 1986), where lordotic behavior occurred despite the suppression of PR induction.

These data seem to indicate that EB-induced PR may not be necessary for the activation of sexual receptivity in mice. In terms of the level of PR present, Oil + P-treated mice did not differ from TAM + EB + P treated mice, although only the latter were capable of lordosis to varying degrees dependent upon the dose of TAM. Therefore, it seems that the antagonistic mechanism of TAM is most likely a blockade of an estrogenic effect other than PR induction.

Species Specific Effects of Tamoxifen

It is clear that the effects of TAM are variable both within and between species. Differences have been noted in the way mice and rats respond to TAM. When TAM is administered alone neither species is sexually receptive. However, in rats, the dose of TAM (2 mg) that is incapable of activating lordosis significantly increased PR in the

HPOA (Etgen & Shammamian, 1986). In mice, however, a dose (100 μg) that did not activate lordosis had no inductive effects on PR. When TAM (2 mg) was administered simultaneously with EB, only minimal suppression of PR induction was exhibited in rats, whereas TAM totally suppressed EB-induced PR induction in all of the doses utilized (5-100 μg) in mice. The fact that data from rats were obtained with a much higher dose of TAM does not seem to explain the disparate effects of TAM in the two species. In addition, the highest dose of TAM used in the present study (100 μg) translates to an approximate dose of 3.3mg/kg body weight. This is close to the 5mg/kg dose which was utilized in rats to suppress E-dependent responses.

Variability of Tamoxifen Action in Mice

In mice, the biochemical component (PR induction) was more sensitive to the antagonistic properties of TAM than the behavioral component (lordosis). Variability of the action of TAM in the CNS of the mouse is evident when one compares the effect of TAM on aggression (Simon & Perry, 1988) with the present data on lordosis. Unlike aggression, where TAM seems to be estrogenic, it was not an agonist for lordosis and the induction of PR in the HPOA within a similar dose range. This response specificity may be attributable to the different neuroanatomical substrates which regulate these behaviors; the septum for aggression (Owen, Peters, & Bronson, 1974; Slotnick & McMullen, 1972)

and the VMN (ventromedial nucleus) for lordosis (Davis, McEwen, & Pfaff, 1979; Howard, Etgen & Barfield, 1984). An additional consideration is that these behaviors are sex-specific; aggression is male-typical and lordosis is female-typical.

Potential Mechanisms Mediating the Variable Action of Tamoxifen

Given the sexual dimorphism in the behavioral systems discussed above, it becomes possible to speculate that one source of differences in the eventual response to TAM may be the different hormonal environments of male and female mice. A recent report described the effect of perinatal and pubertal hormonal environments on the differential availability of the steroid response element (SRE) in peripheral tissue (Chatterjee & Roy, 1990). The SRE is a consensus sequence of DNA that the activated receptor complex binds to regulate gene transcription. The availability of the SRE may be a function of local chromatin structure. Tissue specific availability of the SRE due to the different hormonal environments of the male and female mouse may play a role in the variability of the effect of TAM on aggression and lordosis.

Evidence that suggests a possible role of chromatin organization in hormonal responsiveness necessitates an understanding of the concept of chromatin acceptor sites. Chromatin acceptor sites are the nuclear binding sites for

steroid-receptor complexes. There is much controversy regarding the identity of the acceptor site. Presently, three classes of nuclear acceptor sites have been identified, chromatin acceptor sites, the nuclear matrix, and DNA acceptor sites or SRE (Rories & Spelsberg, 1989). The chromatin acceptor sites consist of specific chromatin proteins bound to DNA which bind steroid receptors in a saturable, high affinity, tissue- and steroid- specific fashion (Alexander, Greene & Barrack, 1987; Spelsberg, Littlefield, Seelke, Martin-Dani, & Toyoda, 1983).

The second class, the nuclear matrix (NM), is defined as the salt- and nuclease- resistant nuclear substructure, comprised of approximately 7% of the total nuclear protein and 2% of the total nuclear DNA. The NM is intimately involved in DNA replication and transcription (Buttyan, Olsson, Sheard, & Kallos, 1983). The NM contains high affinity binding sites for both estrogens and androgens which are diminished following withdrawal of hormonal stimulation (Barrack & Coffey, 1980). These sites display saturable, tissue-specific and steroid-specific binding. Because the NM sites and the chromatin acceptor sites have many similar properties it is thought that the NM may represent the active constituent of the chromatin acceptor sites, and thus represent the same class of acceptor sites (Barrack, 1987; Hora, Horton, Toft, & Spelsberg, 1986).

The third class of nuclear acceptor sites is the

aforementioned SRE, which consists solely of a consensus sequence of DNA which is specific for each receptor species, despite a marked similarity between them. For instance, the SRE for estrogen can be changed into that of glucocorticoids by changing only two base pairs (Martinez, Givel, & Wahili, 1987). The SRE is the only DNA element identified as necessary and sufficient for conferring steroid inducibility on a gene promoter (Rories & Spelsberg, 1989). However, there is controversy as to whether the SRE can account for the biological selectivity of receptor action in vivo, in light of the similarities between various SREs and the low degree of binding selectivity observed in in vitro studies. Binding selectivity is defined as the degree of discrimination by the activated hormone-receptor between specific and nonspecific DNA fragments. In light of reported variable hormonal responsiveness within circumscribed regions of prostatic tissue (Prins, 1989), it is difficult to conceive how the SRE, which is presumably invariant, provides the context for differential regulation given that the receptor species are also presumed to be invariant.

It seems that the most likely candidate for the nuclear acceptor site is the chromatin acceptor that is comprised of both the SRE and various nuclear proteins. It has been suggested that the protein composition of the chromatin may play a role in the effect of the ligand-receptor complex for

that specific acceptor site. Support for the role of chromatin acceptor sites comes from a study in which shark ER was utilized as a probe to examine chromatin binding in different shark and mammalian tissue preparations. The only combination that revealed significant binding was shark testicular ER with shark testicular chromatin. In addition, ER from rabbit uterus, shark oviduct or mouse testis bound minimally to shark testicular chromatin (Ruh, Singh, Mak, & Callard, 1986) indicating that ER-chromatin binding is tissue- and species-specific. Further support comes from examining antiestrogen resistance in cell lines. Comparison of the chromatin binding characteristics of antiestrogen-resistant and -sensitive sublines of MCF-7 cells, subline RR and E-3 respectively, indicated that resistance is a function of chromatin composition (Singh, Ruh, Butler & Ruh, 1986).

Other factors that have been implicated in the variable response of TAM include domains on the receptor itself as well as the presence of transcription factors (Green, 1990; Green & Chambon, 1988; Lees Fawell, & Parker, 1989a, b). ER contains both constituitive and ligand induced transcriptional activating functions (TAF1 and TAF2, respectively; Lees et al, 1989a,b; Tora, White, Brou, Tasset, Webster, Scheer, & Chambon, 1989). It has been postulated that the ability of TAM to antagonize E-dependent processes may stem from its' inability to activate TAF2 (the

ligand-induced TAF). This may be due to the different receptor conformations invoked by TAM and E (Ruh, Turner, Paulson & Ruh, 1990). Variable agonist effects have been ascribed to the differences in the activity of TAF1 in different tissues and species. For example, removal of TAF2 from the receptor produces a constituitively acting transcription factor whose activity represents 1-10 % of the activity seen with the intact human ER or glucocorticoid receptor (GR) (Hollenberg, Giguere, Segui, & Evans, 1987; Kumar, Green, Stack, Berry, Jin, & Chambon, 1987). In the case of the rat GR, though, the activity present represents 50% of the activity of an intact receptor (Godowski, Rusconi, Miesfeld, & Yamamoto, 1987). The ability of TAF1 to synergize with cell and tissue specific transcription factors has also been indicated in the variability of agonist effects. Similar mutants of the human ER (those that lack TAF2) have approximately 5% intact receptor activity in HeLa or COS cells (Bocquel, Kumar, Stricker, Chambon, & Gronemeyer, 1989; Kumar, et al., 1987) but 60-70% activity in chick embryo fibroblasts and 100% activity in yeast (Tora, et al., 1989; White, Metzger, & Chambon, 1988). This variability in TAF1 function in the different cell lines has been taken as evidence for cell-specific differences in presence of transcription factors which, together with the receptor, regulate transcription. Because lordosis and aggression are regulated by different

neuroanatomical regions there exists the possibility that such tissue and/or cell specific variation in the presence of transcription factors may play a role in the variable effects of TAM in male and female behaviors.

CHAPTER FIVE

FUTURE RESEARCH DIRECTIONS

The preceding discussion makes it evident that complex biochemical investigations are necessary to elucidate the molecular mechanisms responsible for the differing actions of TAM in the male and female mouse CNS. For example, intact as well as sequentially deproteinized chromatin from the VMN and the septum of both the female and the male could be assayed with radiolabelled TAM to determine the availability of the SRE in the tissue preparations of the different sexes. An alternative approach to investigating the hormonal regulation of chromatin organization would be the investigation of another male-typical behavior, such as mounting, which is regulated by the medial preoptic area (MPOA) (Christensen & Clemens, 1975; Lisk, 1967). If TAM acts as an agonist for mounting this would provide support for the hypothesis that the availability of SRE for TAM-ER complex in the mouse CNS may be the result of hormonally regulated chromatin organization.

CHAPTER SIX

SUMMARY

In conclusion, TAM did not possess any agonist properties in the CNS of the female mouse for the activation of lordosis or for the induction of PR in the HPOA within the dosage range utilized. Antagonism was evident in the ability of TAM to suppress lordosis as a function of dose, although suppression was never totally complete. However, all of the doses suppressed PR induction to the basal level exhibited by oil-treated mice. When these results are compared to those obtained in a comparable study utilizing rats (Etgen & Shamammian, 1986), clear differences emerge in the biochemical data. Concerning PR induction, TAM was an agonist in rats when administered alone, but when administered simultaneously with EB, TAM acted not as an antagonist, but as a weak agonist evident by only minimal suppression of PR induction.

The effects of TAM vary within mice, dependent upon the response under consideration. This is evident when comparing the results of the present study with the effect of TAM on aggression in intact male mice (Simon & Perry, 1988). TAM potentiated aggression, acting estrogenically, in intact males, while it was solely an antagonist in the female for the responses studied. Due to the sexually dimorphic nature of aggression and lordosis, it was speculated that the agonist/antagonist action of TAM in the

mouse CNS may be a result of differential chromatin organization due to hormonal environment. In light of the species-, tissue-, and even cell- specific activity of transcriptional activating functions (TAF's) localized on ER, it is also plausible that variable TAF activity may also serve as the mechanism for the agonist/antagonist actions of TAM.

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END

OF

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