Effects of Body Weight, Adrenal Status, and Estrogen Priming on Hypothalamic Progesterin Receptors in Male and Female Rats¹

ANNE M. ETGEN²

Department of Biological Sciences, Rutgers University, New Brunswick, New Jersey 08903

Abstract

Some laboratories have reported sex differences in the effect of estrogen priming on neural progesterin receptor concentration, whereas others find no such differences. The present studies sought to identify experimental variables which might differentially influence the measurable level of hypothalamic cytosol progesterin receptors in adult male and female rats. The influence of three variables, body weight, adrenal status, and estrogen priming regimen, was evaluated. Treatment with low doses of estrogen (2 or 8 μ g of estradiol benzoate (EB) 48 hr before sacrifice) was slightly but not significantly more effective in elevating hypothalamic progesterin receptor content in age-matched females than in males despite the 40% greater body weights of males. When the same doses of EB were administered per 200 gm of body weight to animals with and without adrenal glands, both basal and estrogen-induced levels of hypothalamic progesterin receptor were significantly higher in adrenalectomized/gonadectomized rats than in rats which were gonadectomized only, regardless of sex. There was no significant effect of sex or any interaction between sex, adrenal status, or estrogen priming dose. The only significant sex difference in brain progesterin receptors occurred when weight-matched males and females received multiple injections of higher priming doses of estrogen (three daily injections of 10 μ g of EB). Under these conditions, females showed up to 2-fold higher levels of hypothalamic progesterin receptors than males, regardless of adrenal status. These data suggest that sex differences may exist in the neural progesterin receptor systems of male and female rats, but the relationship of these differences to sex differences in neuroendocrine function or behavior is not clear.

Research on sex differences in the central nervous system has expanded rapidly in recent years. Although the list of recognized sexual dimorphisms in brain anatomy and chemistry has lengthened considerably, the relationship of such differences to sex differences in animal physiology generally remains unclear. Shortly after the unequivocal demonstration that rodent brain contains specific, sat-

urable, high affinity progesterin-binding macromolecules (receptors; Kato and Onouchi, 1977; Moguilewsky and Raynaud, 1977), sex differences in neural progesterin receptors were reported. Moguilewsky and Raynaud (1979) found that estrogen priming, which activates estrous behavior in female but not in male rats, was less effective in elevating hypothalamic-preoptic area (HPOA) progesterin receptor levels in males than in females. Neither these authors nor Thrower and Lim (1980) observed sex differences in brain progesterin receptor levels between gonadally intact males and ovariectomized females which had not been treated with estrogen. More recently, Rainbow et al. (1982) reported that castrated, estrogen-primed male rats have fewer cytosol progesterin receptors in the ventromedial nucleus (VMN) of the hypothalamus and periventricular region of the preoptic area than do similarly treated females. These data have led the authors to suggest that the differential expression of estrous behaviors in male and female rats might result from a deficiency in estrogen induction of HPOA progesterin receptors in males.

Another laboratory, however, has reported that estrogen greatly increases cytosol progesterin receptor levels in hypothalamus of castrated male rats (Naess and Attramadal, 1978). Still other laboratories have found that estrogen priming produces comparable increases in HPOA progesterin receptor levels in normal females, normal males, and neonatally androgenized or estrogenized females (Etgen, 1981; Ghraf et al., 1983; Kirchoff et al., 1983). Of these four groups of rats, only normal females show high levels of estrous behavior in response to estrogen plus progesterin treatment in adulthood. These data question the relationship between estrogen induction of HPOA progesterin receptors and the differential sensitivity of male and female rats to the estrous behavior-facilitating properties of estrogen.

In view of the large discrepancies in estrogen inducibility of brain progesterin receptors in male and female rats reported by independent laboratories and the consequent interpretation of such data in terms of sex differences in brain responsiveness to estrogen stimulation, it seemed important to reinvestigate the induction of HPOA progesterin receptors by estrogen in the two sexes. More specifically, the present experiments sought to identify factors other than sex which might influence the measurement of brain cytosol progesterin receptor content in male and female rats.

Materials and Methods

General methods. Sprague-Dawley rats obtained from Blue Spruce Farms, Inc. (Altamont, NY) or Harlan Sprague-Dawley (Indianapolis, IN) were used throughout. Animals were housed by sex in groups of two to four and were maintained on a 12-hr/12-hr reversed light/dark cycle (lights off at 9:30 AM) with food and water available *ad libitum*. Bilateral gonadectomy and/or adrenalectomy was performed under Metofane anesthesia (Pitman-Moore, Inc., Washington Crossing, NJ). Adrenalectomized animals were given 1.0% NaCl in their drinking water up until the time of sacrifice. All hormone injections were given subcutaneously in peanut oil vehicle.

Materials. The steroid 17- β -estradiol benzoate (EB) was purchased from Steraloids, Inc. (Wilton, NH) and dissolved in peanut oil prior to injection. The radiolabeled progesterin R5020 (17 α -[methyl-³H]R5020; specific activity, 87

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² To whom correspondence and reprint requests should be sent, at her present address: Department of Psychiatry, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

Ci/mmol) and unlabeled R5020 were obtained from New England Nuclear Corp. (Boston, MA) and checked for purity by thin layer chromatography before use and at regular intervals throughout the experiment. All other chemicals were of reagent grade.

Progesterin receptor assays. Animals were sacrificed by decapitation, and the entire HPOA was removed as described previously (Etgen, 1979). Cytosol progesterin receptor levels were assayed in freshly dissected tissue using a modification of the method of Ginsburg et al. (1974). Pooled tissue from two or three animals was homogenized in ice-cold buffer (10 mM Tris-HCl, pH 7.4 at 0°C; 1.5 mM Na₂EDTA; 12 mM monothio glycerol; 10% (v/v) glycerol; TEMG) in Teflon-glass tissue grinders. The homogenate was centrifuged at $850 \times g$ for 10 min, and the resulting supernatant was recentrifuged at $105,000 \times g$ for 1 hr. The clear high speed supernatant (cytosol) was removed and used immediately. Aliquots (150 or 200 μ l) of cytosol were incubated at 0°C for 5 hr with radiolabeled R5020 in the presence or absence of 1,000-fold excess unlabeled R5020. For one-point assays, the final subsaturating concentration of [³H]R5020 was 0.5 nM. For saturation analyses, the [³H]R5020 concentration in the incubations ranged from 0.1 to 3 nM. Bound [³H]R5020 was separated from free [³H]R5020 by applying samples of each incubate (200 μ l) to individual minicolumns (0.5 $\times$ 9 cm) of Sephadex LH-20 equilibrated in TEMG. Samples were washed in with 200 μ l of TEMG, and bound radioactivity was eluted in the first 0.8 ml following the void volume directly into scintillation minivials. Scintillation fluid (5 ml of Tritonol) was added, and radioactivity was counted in a Beckman LS-100C liquid scintillation counter with an automatic external standard. Specific binding was calculated by subtracting nonspecific binding (with excess unlabeled R5020) from total binding (without excess unlabeled R5020).

Results

Effects of body weight. Since adult male rats are considerably heavier than females of the same age, it is possible that age-matched males and females would show different levels of estrogen-induced progesterin receptors because males receive a lower effective hormone dose if body weight is not considered at the time of estrogen administration. Therefore, the first experiment measured estrogen induction of HPOA cytosol progesterin receptors in age-matched adult (65 to 100 days old) male and female rats which differed by approximately 40% in total body weight (range, 30 to 46%). Animals which had been gonadectomized for at least 4 days received 2 or 8 μ g of EB or oil vehicle and were sacrificed 48 hr later for determination of progesterin receptor levels. In four independent replications, vehicle-injected males and females showed comparable levels of receptor (4 to 5 fmol/mg of protein). Figure 1 shows that females given 2 μ g of EB had 11% greater induction of HPOA

Figure 2. HPOA cytosol progesterin receptor levels in gonadectomized (adrenals intact) and gonadectomized/adrenalectomized male and female rats given oil vehicle, 2 or 8 μ g of EB/200 gm of body weight. Hatched bars, females; open bars, males. Analysis of variance revealed significant differences as a function of adrenal status and estrogen priming dose (see the text for details). Each value represents the mean of five to seven independent replications.

cytosol progesterin receptors than did age-matched males; at the 8- μ g dose, EB induced 21% more receptor in females than in males. Thus, females tended to show slightly greater elevations in brain progesterin receptor levels in response to low doses of EB given as a single injection, but neither of these differences was statistically significant (*t* tests, $p > 0.05$). Therefore, it is likely that body weight differences in age-matched males and females do not account for the observation of sex differences in neural progesterin receptors by some laboratories (Mogilewsky and Raynaud, 1979; Rainbow et al., 1982). However, since there was a small but consistent difference between the sexes, EB was administered relative to body weight in the remainder of the experiments.

Effect of adrenal status. It has been suggested that the increased release of adrenal steroids following castration in male rats might interfere with measurement of progesterin receptor levels in males due either to competition by endogenous steroids for cytosol-binding sites or to translocation of cytosol receptors to the nuclear compartment (Etgen, 1981, 1984). Scatchard analysis of HPOA cytosol progesterin receptors in castrated and castrated/adrenalectomized males suggested that, although the affinity of [³H]R5020 for binding

Figure 1. Induction of HPOA cytosol progesterin receptors by 2 or 8 μ g of EB in age-matched male and female rats which differed by 40% in body weight. Data are expressed as percentage of increase compared to vehicle-injected controls, and each value represents the mean of four independent replications.

Figure 3. Induction of HPOA cytosol progesterone receptors by three daily injections of 10 μ g of EB in weight-matched male and female rats. Data are expressed as percentage of increase compared to vehicle-injected controls, and each value represents the mean of four to five independent replications. *, $p < 0.025$, t tests.

sites did not differ between the two groups, the maximum number of binding sites was always greater in rats that had been both castrated and adrenalectomized (data not shown). Thus, the following experiments evaluated both basal progesterone receptor levels and estrogen induction of HPOA progesterone receptors in gonadectomized and gonadectomized/adrenalectomized males and females. The weights of animals ranged from 180 to 220 gm, and EB injections were administered as 2 or 8 μ g/200 gm of body weight 48 hr before sacrifice. Adrenalectomies were performed 72 hr before sacrifice.

Figure 2 shows the level of HPOA cytosol progesterone receptors, in femtomoles per milligram of protein, measured in gonadectomized and gonadectomized/adrenalectomized male and female rats primed with oil vehicle or 2 or 8 μ g of EB. The data were subjected to a three-way analysis of variance (sex $\times$ adrenal status $\times$ EB dose) using an SAS GLM procedure and least squares means for planned post hoc comparisons (SAS User's Guide, 1979). Significant differences were found as a function of adrenal status ($F = 9.59$; $df = 1,58$; $p < 0.003$) and EB dose ($F = 26.58$; $df = 2,58$; $p < 0.0001$). There were no significant sex differences or any significant interactions between sex, adrenal status, and EB dose. Thus, adrenalectomized rats had higher measurable brain cytosol progesterone receptor levels regardless of sex or estrogen priming. As suggested by the preliminary Scatchard analyses, vehicle-injected control males had 45 to 60% more available progesterone-binding sites when they were both castrated and adrenalectomized than when they were only castrated. Ovariectomized females also had higher basal levels of neural progesterone receptors when they were adrenalectomized, but the difference was somewhat less than in males (30 to 40%). In addition to the adrenal effects, post hoc pairwise comparisons indicated that 8 μ g of EB induced significantly higher levels of progesterone receptors than did 2 μ g, regardless of sex or adrenal status (least squares means, $p < 0.02$) and that both 2 and 8 μ g of EB significantly elevated receptors in comparison with vehicle controls (least squares means, $p < 0.0001$).

Effects of estrogen priming regimen. Results of the first two experiments indicate that the observation of sex differences in HPOA

progesterone receptor induction by some investigators but not others cannot be attributed entirely to procedural differences such as failure to control for body weight or adrenal status. The most robust sex difference in estrogen induction of brain progesterone receptors was reported by Rainbow et al. (1982). Those investigators used EB priming procedures (10 μ g/day for 3 days before sacrifice) different from the ones used in the present study. Therefore, a final experiment evaluated the effects of the EB priming procedure on progesterone receptor induction in gonadectomized and gonadectomized/adrenalectomized male and female rats. Animals were adrenalectomized 4 days before sacrifice, and estrogen priming was the same as that reported by Rainbow et al. (1982), i.e., 10 μ g/day for 3 days before sacrifice. Control rats received parallel injections of oil vehicle, and all animals were sacrificed 24 hr after the last injection.

As shown in Figure 3, significant sex differences in estrogen induction of HPOA cytosol progesterone receptors can be observed when animals receive more prolonged priming with higher EB doses. In gonadectomized animals with intact adrenals, the magnitude of progesterone receptor induction was twice as high in females as in males ($t = 6.88$, $df = 7$, $p < 0.005$). Although the sex difference was not as great in adrenalectomized animals (females = 181% increase; males = 133% increase), it was still statistically significant ($t = 2.52$, $df = 8$, $p < 0.025$). Control levels of HPOA progesterone receptor were comparable to those shown in Figure 2.

Discussion

These results indicate that at least two variables other than sex, namely, adrenal status and estrogen priming dose, may influence the measurement of brain cytosol progesterone receptors in adult rats. A third variable, body weight, appeared to play a minor or negligible role. Despite the 40% greater body weights of male rats when compared to age-matched female counterparts, there was no significant sex difference in the level of progesterone receptor induced in the HPOA by 2 or 8 μ g of EB.

Adrenal effects on neural progesterone receptors are more complicated. The second experiment demonstrated that when body weight was controlled, adrenalectomy significantly increased both basal and estrogen-induced levels of HPOA progesterone receptors in both sexes. This result is consistent with earlier observations of increased brain uptake of progesterone in adrenalectomized rats (Whalen and Luttge, 1971). However, in the third experiment, which used much higher priming doses of EB, the induced level of progesterone receptor was consistently lower in adrenalectomized animals. Thus, it appears that the adrenal gland can modify profoundly the measurement of brain progesterone receptors, but the underlying mechanism is not obvious. We cannot mimic the effect of adrenalectomy by treating animals with metyrapone, an inhibitor of adrenal steroid biosynthesis, or by suppressing ACTH with high doses of dexamethasone (A. M. Etgen, unpublished observations). However, we have been unable to measure blood levels of steroids following drug treatments; thus, we cannot be certain that the metyrapone or dexamethasone effectively reduced adrenal steroid production and release. Therefore, it is not known whether the elevated progesterone receptor levels in adrenalectomized rats reflect the loss of endogenous adrenal steroids which could compete for available cytosol-binding sites and/or translocate receptor into the nucleus, or whether the adrenal regulates neural progesterone receptors by a mechanism independent of (or in addition to) its steroid output. Although many questions remain to be answered regarding the role of the adrenal in modulating HPOA progesterone receptors, the present data do lead to the conclusion that the use of adrenalectomized rats by some investigators (Etgen, 1981; Ghraf et al., 1983; Kirchoff et al., 1983) does not account for their failure to detect sex differences in estrogen-induced progesterone receptors.

The only condition which produced reliable, robust sex differences in HPOA cytosol progesterone receptors in the present study was prolonged administration of higher doses of estrogen (10 μ g of EB/day for 3 days). With this estrogen priming regimen, female rats had

consistently higher concentrations of HPOA progesterin receptors than did their male counterparts. Comparable differences were never seen with lower doses of estrogen administered as a single injection, even when body weight and adrenal status were manipulated. Thus, it appears that the most important determinant of sex differences in estrogen induction of HPOA progesterin receptors is the estrogen priming regimen. Single injections of relatively low doses of estrogen (2 and 8 μg of EB), which are sufficient to facilitate estrous behavior in females but not males (Whalen et al., 1971; Whalen, 1974), do not produce significant sex differences in HPOA cytosol progesterin receptors when administered to age- or weight-matched animals (see Figs. 1 and 2). It is not clear whether the observation of a significant sex difference in the final experiment resulted from the higher total dose of estrogen (30 μg of EB) or from the timing of the injections (three daily injections of 10 μg each). The data of Moguilewsky and Raynaud (1979) do not clarify this question. Although they administered single injections of 1 to 100 μg of EB to males and females 40 hr before sacrifice, they did not provide statistical comparisons between the sexes at any dose level. It should also be noted that the latter authors used a procedure to assay cytosol progesterin receptors (dextran-coated charcoal method) that was radically different from either the present report or that of Rainbow et al. (1982), making it more difficult to compare the results of these three studies.

An important procedural difference between the present experiments and those of Rainbow et al. (1982) is neuroanatomical specificity. The latter study quantified cytosol progesterin receptor levels in individual, microdissected nuclei of the rat brain rather than in grossly dissected tissue blocks as used in the present study. Therefore, the results of the two studies are not directly comparable. For example, it is conceivable that sex differences would have been noted in our first two experiments specifically in the VMN and periventricular preoptic area had we assayed microdissected tissue. Perhaps the trend toward lower levels of estrogen-inducible receptors in age-matched males (see Fig. 1) would have been attributable entirely to differences in these two nuclei. However, it is also possible that microassay of cytosol progesterin receptor content in hypothalamic and preoptic nuclei from rats given much lower doses of estrogen would fail to reveal significant sex differences when both body weight and adrenal status were controlled. The only other report of estrogen induction of cytosol progesterin receptors in microdissected brain nuclei (Parsons et al., 1982) used even higher priming doses of EB (20 $\mu\text{g}/\text{day}$ for 3 days). Thus, it is difficult to evaluate whether the comparatively reduced level of progesterin receptors found in the VMN of estrogen-primed males is less than, equal to, or greater than the concentration which would be induced in females by low but behaviorally active estrogen doses.

Results of this study suggest that the physiological significance of reported sex differences in the brain progesterin receptor system must be interpreted cautiously. Although it is clear that the neural progesterin receptor systems of male and female rats do not always respond in the same way to estrogen priming, it is not clear how these sex differences relate to any known sexual dimorphisms in neuroendocrinology or behavior. Since behaviorally effective doses of EB given as a single injection can produce comparable levels of induced progesterin receptor in the HPOA of males and females, it

may be premature to speculate that the insensitivity of male rats to the estrous behavior-promoting actions of ovarian steroids are causally related to the insensitivity of their neural progesterin receptor system to stimulation by estrogen. In fact, it is possible that the differences observed in this study may be entirely unrelated to sexual dimorphisms in brain responsiveness to estrogen. Rather, they may reflect sex differences in liver metabolism of steroids (for a recent review see Gustafsson et al., 1983) or in progesterin receptor translocation (Kato et al., 1984), nuclear retention, or processing.

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