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Serotonin Binding Sites During Proestrus and Following Estradiol Treatment

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WILLIAMS, J. AND L. UPHOUSE. *Serotonin binding sites during proestrus and following estradiol treatment.* PHARMACOL BIOCHEM BEHAV **33**(3) 615–620, 1989.—The binding of ^{3}H-5-HT to hypothalamic, hippocampal and striatal membranes from female rats was examined. During the day of proestrus, there was a significant increase in binding from morning to evening. The increase reflected changes in both K_d and B_{max}. Effects of estradiol were examined in ovariectomized rats, and changes in ^{3}H-5-HT binding resulted from an increase in the K_d after estradiol treatment. These in vivo effects of estradiol were not seen when tissue was incubated in vitro with estradiol. However, the compound, polyvinylpyrrolidone, used in the in vitro incubation significantly increased the K_d for binding to ^{3}H-5-HT and estradiol attenuated the increase. The potential significance of these changes to serotonin's modulation of reproductive function is discussed.

Serotonin Proestrus Estradiol Female Rat

IN hypothalamic and associated brain areas, gonadal steroids exert a variety of actions on neural activity which contribute to the hormones' regulation of neuroendocrine function and sexual behavior (24, 25, 30, 31). Many effects of steroids on neural tissue, delayed in onset and prolonged in duration, involve intracellular receptors operating via the genome (24,31). Brain areas in which such responses occur are those characterized by high densities of intracellular steroid receptors (33,38). However, gonadal steroids can also exert rapid effects on the nervous system and these fast responses occur in regions containing intracellular receptors and also in regions lacking intracellular receptors (23). These additional effects of steroids may occur through (a) direct effects of the steroid on membrane integrity (b) modulation of neurotransmitter release, reuptake or postsynaptic action or (c) as secondary effects of the hormones on regions rich in intracellular steroid receptors.

In the human female, a relationship has been inferred between gonadal hormones and symptoms of anxiety and depression (8, 29, 45). Affective disorders are more frequent in women than in men (43) and one of the adverse reactions to oral contraceptives is depression (15,16). In rats, sex differences are evident in the metabolism and sensitivity to the antidepressant imipramine (18–20, 44). Estradiol increases ^{3}H-imipramine binding in male rat frontal cortex, (35) and increased ^{3}H-imipramine binding has also been seen in ovariectomized rats treated for 12 days with estradiol. In ovariectomized rats, increased ^{3}H-serotonin uptake coincided with the increased imipramine binding (36).

Interest in neuroendocrine function and affective disorders has precipitated a renewed investigation of neurotransmitter responses to gonadal steroids. Although neurotransmitter effects of gonadal steroids are well established for areas involved in neuroendocrine control (6, 7, 10, 22, 34), they are less well characterized for other brain regions. Treatment of ovariectomized rats with estradiol produced a rapid decrease in cortical ^{3}H-5-HT binding, presumably reflecting 5-HT_1 receptors (3). Two weeks exposure also decreased the binding of ^{3}H-serotonin and ^{3}H-dihydroalprenolol, indexing β-adrenergic receptors, but increased ^{3}H-spiroperidol binding with mianserin as competitor, presumably reflecting 5-HT_2 receptors (4).

Since cortical tissue contains few, if any, intracellular estradiol receptors (33), it is unlikely that the cortical binding changes reflect the hormone's interaction with cortical intracellular receptors. Hormone induced modulation of cortical neurotransmitter receptors could, however, reflect altered neurotransmitter activity in axon terminals of steroid concentrating somata located in noncortical locations. Reduced cortical 5-HT levels (when 5-HT_2 receptors were elevated and 5-HT_1 receptors were decreased) seen after treatment of ovariectomized rats for 2 weeks with estradiol (4) is consistent with such a possibility. However, decreases in ^{3}H-5-HT binding have also been observed when cortical membranes were incubated in vitro for 2 hours with estradiol (3,5). It was suggested that the acute decrease in 5-HT_1 binding observed in vivo could result from estradiol acting directly on cortical membranes.

There have been few attempts to identify cortical neurotransmitter receptor changes resulting from physiological variations in levels of gonadal steroids. Biegon and Samuel (5) reported modulation of ^{3}H-5-HT binding in steroid concentrating areas but were unable to demonstrate differences among estrous, proestrous, or diestrous females in cortical ^{3}H-5-HT binding. Similarly,

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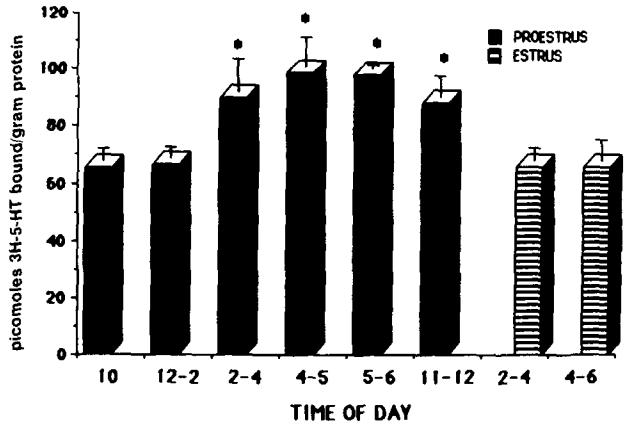

FIG. 1. Hypothalamic ^{3}H-5-HT binding during proestrus. Regularly cycling, adult female rats were sacrificed at various times during the days of either proestrus or estrus. Hypothalamic membranes were used for ^{3}H-5-HT binding as described in the Method section. Data are the mean ± S.E.M. of ^{3}H-5-HT specifically bound per gram membrane protein for 3–4 animals a each time point. Asterisks indicate a significant difference ($p<0.05$) from both proestrous (10–12 a.m.) and estrous (2–4 p.m.) time points.

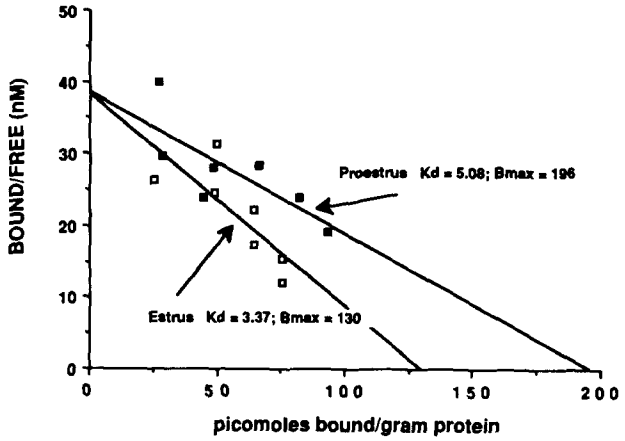

FIG. 2. Scatchard analysis of hypothalamic proestrous and estrous ^{3}H-5-HT binding. Figure 2 shows the effects of Scatchard comparisons between estrous and proestrous females sacrificed between 6 and 7 p.m. Hypothalami of 10 animals from each stage of the cycle were used for membrane preparation. The figure shows the specific binding for a concentration range from 0.5 to 12 nM ^{3}H-5-HT. Each data point represents an average of triplicate determinations. The entire analysis was repeated a second time with similar results.

Rehavi *et al.* found no differences among diestrous, proestrous or estrous females even though such changes were evident in ovariectomized females treated with estradiol (36). In a previous study, we identified changes in cortical serotonin binding during the female rat estrous cycle (41). In the reports of both Biegon and Samuel and of Rehavi *et al.*, binding was compared in females sacrificed at a single time point on the various days of the cycle and this may have accounted for failures to observe cortical changes during the estrous cycle. Our findings demonstrated that cortical ^{3}H-5-HT binding was low on the morning and early afternoon of proestrus. Binding then increased during the late afternoon and evening of proestrus and remained elevated during most of the next day (estrus). These results were consistent with estradiol's putative ability to decrease ^{3}H-5-HT binding and led to the suggestion that the proestrous increase in binding resulted from an estrogen withdrawal during the proestrus to estrus transition.

In the following manuscript, we have extended the investigation of proestrous changes in ^{3}H-5-HT binding to include hypothalamic, striatal and hippocampal tissue and have further evaluated the effects of in vivo and in vitro exposure to estradiol on ^{3}H-5-HT binding.

METHOD

Materials

Labeled serotonin [5-(1,2-^{3}H-(N)-serotonin, creatine sulfate, 29.5 Ci/mmol)] was obtained from New England Nuclear (Boston, MA). Unlabeled serotonin (5-HT, creatine sulfate), ascorbic acid, pargyline, estradiol 17-β, polyvinylpyrrolidone (PVP; average mol.wt. = 40,000) and sesame oil were obtained from Sigma Chemical Company (St. Louis, MO). Betaphase was purchased from WestChem (San Diego, CA). All other supplies came from Fisher Scientific Supply Company (Springfield, NJ).

Animals and Housing Conditions

Regularly cycling, adult female rats (CDF-344) or females ovariectomized as adults were used in the study. Females were born in the laboratory from breeding stock obtained from Charles River Laboratories (Kingston, NY facility). Animals were weaned at 21 days of age and housed with like-sexed littermates for the remainder of the experiments. Animals were housed 4 or 5 per cage in hanging cages in a colony room with a 12-12 hour light-dark cycle with lights on at 7:00 a.m. (CDT). Food and water were available ad lib. When ovariectomy was performed, adult rats were anesthetized with Nembutal and the ovaries were removed by bilateral incision. Rats received a single injection of 10 μg estradiol (SC in sesame oil) one week later and were used for the experiments the following week.

Vaginal Smears

Vaginal smears, taken between 8 and 10 a.m., were monitored as previously described (39) for at least 2 complete estrous cycles beginning at 60 days of age. Smears with nucleated or a combination of nucleated and cornified cells, but an absence of leucocytes, were judged as proestrous smears. The preceding smear history must also have predicted a proestrous state before the female was included in the study. Estrous females showed predominantly cornified cells and must have shown a proestrous smear on the preceding day.

Treatment of Animals

When binding parameters were investigated in intact females, rats were sacrificed by decapitation at various times during the days of estrus or proestrus. Cortex, hypothalamus, striatum, and hippocampus were removed by the procedure of Glowinski and Iversen (12) and were rapidly frozen on dry ice. For studies with ovariectomized females, rats were injected with 10 μg estradiol (10 μg/0.1 ml sesame oil; SC) for either one or two days. Rats were sacrificed 1) 3 hours after the first injection, 2) 3 hours after the second injection, 3) 24 hours after the second injection, or 4) 48 hours after the second injection. The hypothalamus plus

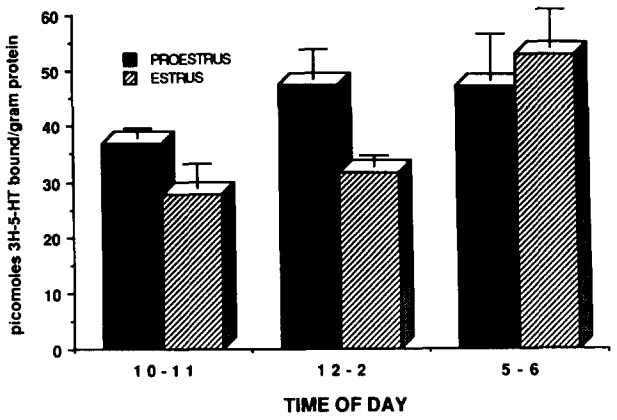

FIG. 3. ^{3}H-5-HT binding to hippocampal membranes during the estrous cycle. Adult female rats were sacrificed at the indicated times on either proestrus or estrus. The hippocampi were used for ^{3}H-5-HT binding. Aliquots of the membranes (equivalent to 10 mg original tissue) were incubated in triplicate with 3 nM ^{3}H-5-HT plus or minus 10^{-6} M unlabelled 5-HT as competitor. Data are expressed as mean ± S.E.M. picomoles ^{3}H-5-HT specifically bound per gram membrane protein. Data are the means respectively for 10, 8, and 7 proestrous females and 6, 7, and 6 estrous females.

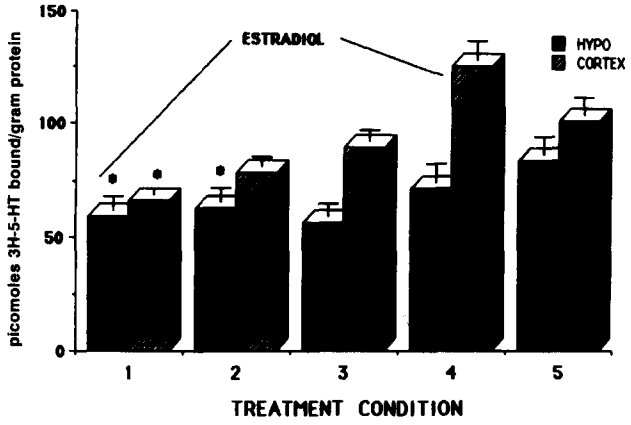

FIG. 4. Hypothalamic and cortical ^{3}H-5-HT binding after estradiol treatment of ovariectomized rats. Ovariectomized rats were assigned to one of the following treatment conditions: 1) 10 μg estradiol—sacrifice 3 hours later; 2) 10 μg estradiol on each of 2 consecutive days—sacrifice 3 hours afte second injection; 3) 10 μg estradiol on each of 2 days—sacrifice 24 hours after second injection; 4) 10 μg estradiol on each of 2 days—sacrifice 48 hours after second injection; 5) vehicle control. Hypothalamus and frontal cortex were used for ^{3}H-5-HT binding. Data are means ± S.E.M. respectively for hypothalamus from 7, 7, 8, 7, and 11 animals and for cortex from 6, 6, 7, 8, and 11 animals in each condition. Asterisks indicate a significant difference from the appropriate control tissue.

preoptic area and frontal cortex were removed as described by Glowinski and Iversen (12). Tissue was frozen on dry ice and stored at −60°C until preparation of membranes. Hypothalamic tissue from individual animals was used for single concentration ^{3}H-5-HT binding. Cortical tissue was used for both single point binding studies and for Scatchard analyses (37).

Preparation of Membranes and ^{3}H-5-HT Binding

Membranes, prepared as previously described (1), were used for ^{3}H-5-HT binding as described by Peroutka and Snyder (32) and as previously performed in our laboratory (40). Tissue, diluted in 19 volumes of solution, was subjected to repeated centrifugations to obtain the crude membrane preparation. The membrane pellet was resuspended in 40 mM tris, pH 7.4, and was incubated for 10 minutes at 37°C. After one further centrifugation, the pellet was suspended in the binding buffer and was frozen at −20°C. The frozen tissue was thawed gently and membranes from 10 mg original tissue (about 300 μg membrane protein) were incubated for 10 minutes in 1.0 ml buffer (40 mM tris, pH 7.4 containing 4 mM $CaCl_2$, 10 μM pargyline and 5.7 mM ascorbic acid) with ^{3}H-5-HT (2–3 nM for single point analyses and 0.5 to 12 nM for Scatchard analyses). Incubations were performed in triplicate and parallel triplicates contained 10^{-6} M unlabeled 5-HT for assessment of nonspecific binding. Specific binding was determined as total binding minus that in the presence of competitor and was corrected for membrane protein, determined by the method of Lowry *et al.* (21).

In Vitro Effects of Estradiol

Membranes were made from whole forebrain of intact females as described above. Prior to binding studies, membranes were suspended in 40 mM tris, pH 7.4, 3.5% PVP in tris, or 3.5% PVP in tris plus 10^{-5} M estradiol and were incubated for 2 hours at 24°C. Membranes were then centrifuged at 18,000 × g, resuspended into binding buffer, and used immediately for ^{3}H-5-HT binding as described in the preceding section. Data were analyzed by the method of Scatchard.

Statistical Methods

Results were evaluated by analyses of variance followed by Tukey comparisons of multiple group means or by Dunnett's test for comparison to a single control group. When only two groups were involved, Student's *t*-test was used (46).

RESULTS

Time-Dependent Proestrous Changes

Hypothalamic ^{3}H-5-HT binding during proestrus and estrus is shown in Fig. 1. Similar to our previous report with cortical tissue (41), hypothalamic ^{3}H-5-HT binding changed during the day of proestrus, ANOVA; $F(7,21) = 4.06$, $p \leq 0.006$. Late afternoon and evening of proestrus were significantly different from early morning proestrous and from the estrous time points (all $q \geq 4.8$, $p \leq 0.05$).

Additional animals were used to compare, by Scatchard procedures, ^{3}H-5-HT binding of the hypothalamus from the evening (6–7 p.m.) proestrous and estrous females (see Fig. 2). Because of the large numbers of animals needed (10 per Scatchard) for the analyses of hypothalamic tissue, the procedure was performed only two times. These two analyses show higher B_{max} and K_d in the proestrous females and are consistent with previous observations of a higher K_d and B_{max} for ^{3}H-5-HT binding to frontal cortex of proestrous rats (41).

Striatal and hippocampal membranes were also examined for the proestrus to estrus transition in ^{3}H-5-HT binding. ANOVA of hippocampal binding (see Fig. 3) demonstrated significant effects of killing time, $F(2,39) = 4.9$, $p \leq 0.013$, but stage of the cycle was not significant, $F(1,39) = 3.8$, $p \leq 0.062$. During both days, ^{3}H-

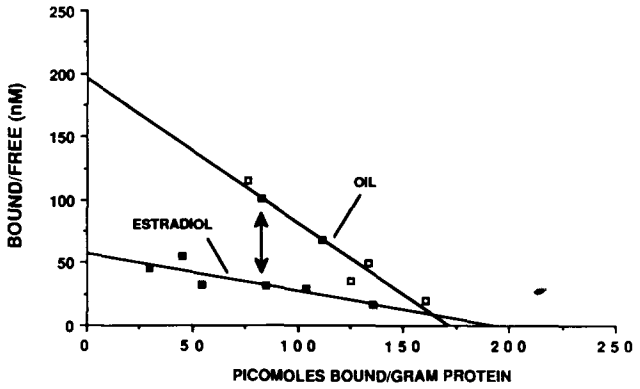

FIG. 5. Effects of estradiol on ^{3}H-5-HT binding in frontal cortex. Ovariectomized females were injected with 10 μg estradiol or oil and were sacrificed 2 hours later. Specific binding of ^{3}H-5-HT (0.5 to 12 nM) $\pm 10^{-6}$ M 5-HT was evaluated by the method of Scatchard. Data points are the average of triplicate determinations. The double arrow marks the concentration of ^{3}H-5-HT used in the single point determinations.

5-HT binding increased. The increase occurred slightly earlier in proestrous than in estrous females and produced a marginally significant interaction between time of day and stage of the estrous cycle ($p=0.062$). No differences were present in striatal tissue either as a function of the time of day or of the estrous cycle (data not shown).

In Vivo Treatment With Estradiol

The effect of estradiol on ^{3}H-5-HT binding of hypothalamic-preoptic area and frontal cortical tissue of ovariectomized rats is shown in Fig. 4. In hypothalamus, analysis of variance indicated a significant effect of treatment, $F(4,35)=2.96$, $p\leq 0.05$. Animals sacrificed 3 hours following a single treatment with estradiol, $q(35,4)=2.57$, $p\leq 0.05$, and 3 hours after two treatments with estradiol, $q(35,5)=2.96$, $p\leq 0.05$ were significantly different from the oil control. By 24 hours following the second treatment with estradiol, ^{3}H-5-HT binding was still lower than the oil control but the difference failed to reach statistical significance, $q(35,3)=2.25$, $p\geq 0.05$. Forty-eight hours following the second estradiol treatment, ^{3}H-5-HT binding was no longer different from the oil control ($p\geq 0.05$).

Estradiol also decreased cortical binding, $F(4,33)=10.04$, $p\leq 0.001$. Animals sacrificed 3 hours following either one or two injections of estradiol were significantly different from the control, respectively $q(33,2)$ and $q(33,3)=3.62$ and 2.30, $p\leq 0.05$, but by 24 hours following the second treatment, estradiol-treated animals were no longer different from the controls ($p>0.05$). Forty-eight hours after the second injection, cortical ^{3}H-5-HT binding was higher than the oil control, $q(33,2)=2.75$, $p\leq 0.05$.

A representative Scatchard comparison between ovariectomized females treated with oil or estradiol and sacrificed 2 to 3 hours later is shown in Fig. 5 and the average results from seven such comparisons are shown in Table 1. In contrast to the expectations from the single point binding, the B_{max} was not significantly altered by estradiol, $t(12)=2.053$, $p=0.063$, but the K_d was significantly larger in the estradiol-treated animals, $t(12)=2.3$, $p=0.038$.

The double arrow shown in Fig. 5 marks the approximate binding concentration of ^{3}H-5-HT which was used in the single point studies and indicates why the single point studies showed reduced ^{3}H-5-HT binding following treatment with estradiol.

TABLE 1

SCATCHARD RESULTS OF ^{3}H-5-HT BINDING TO CORTICAL MEMBRANES FOLLOWING IN VIVO ESTRADIOL TREATMENT

	Mean ± s.e.m.	
Group	K_d (nM)	B_{max} (picomoles bound/gram protein)
Oil	2.61 ± 0.59	174 ± 11
Estradiol	4.73 ± 0.67*	242 ± 31

Ovariectomized female rats were decapitated 2 hours following treatment with 10 μg estradiol or oil. Cortical membranes were used for ^{3}H-5-HT binding as described in the Method section.

*Significantly different from oil control ($p<0.05$).

In Vitro Effects of Estradiol on Cortical ^{3}H-5-HT Binding

In view of the prior observations for increments in the K_d following in vivo treatment with estradiol, it was important to evaluate the direct in vitro effects of the steroid. In the following study, membranes from frontal cortex of intact females were incubated for 2 hours with 10^{-5} M estradiol and were then

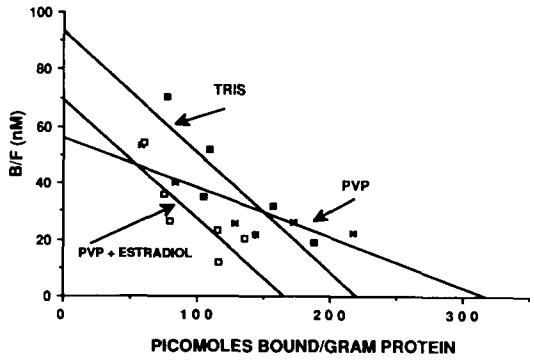

FIG. 6. In vitro effects of estradiol on ^{3}H-5-HT binding. Membranes from whole forebrain of female rats were incubated with tris, PVP, or PVP plus 10^{-5} M estradiol as described in the Method section. Data are plotted by the method of Scatchard. Each point represents the average of 3 triplicate determinations. The entire experiment was repeated three times.

TABLE 2

SCATCHARD RESULTS OF ^{3}H-5-HT BINDING TO CORTICAL MEMBRANES FOLLOWING INCUBATION IN VITRO WITH ESTRADIOL

	Mean ± s.e.m.	
Group	K_d (nM)	B_{max} (picomoles/gram protein)
Tris	2.80 ± 0.20*	224 ± 7
PVP	5.06 ± 0.46	270 ± 44
PVP+Estradiol	2.96 ± 0.28*	209 ± 27

Membranes from whole forebrain of female rats were incubated with tris, PVP, or PVP plus estradiol as described in the Method section.

*Significantly different from membranes incubated with PVP ($p=0.005$).

examined for ^{3}H-5-HT binding by Scatchard comparisons. A representative Scatchard comparison for membranes incubated for 2 hours with tris, polyvinlypyrrolidone (PVP), or PVP plus estradiol is shown in Fig. 6. The mean K_d and B_{max} for three such comparisons are shown in Table 2. Analysis of variance indicated a significant treatment effect for K_d, $F(2,6) = 14.009$, $p = 0.005$ but not for B_{max}, $F(2,6) = 1.116$, $p = 0.387$. The K_d for binding of ^{3}H-5-HT was increased by preincubation with PVP and this was reduced when estradiol was included during the incubation.

DISCUSSION

Three major observations were made in these studies:

1) In hypothalamus, ^{3}H-5-HT binding increased during the day of proestrus. This change, seen in the present paper, is similar to that previously reported for cortex (41). Therefore, tissues with and without high densities of estradiol receptors show similar proestrous changes in ^{3}H-5-HT binding.

2) In vivo treatment with estradiol led to a reduction in the binding of 2–3 nM ^{3}H-5-HT to cortical and hypothalamic tissue. Scatchard analyses suggested that the decreased binding was due to a reduced affinity for the ligand by membranes from estradiol-treated animals.

3) Polyvinylpyrrolidone (PVP), a compound used in previous reports of in vitro effects of estradiol, increased the K_d for binding of cortical membranes to ^{3}H-5-HT and estradiol attenuated the increase by PVP.

In agreement with conclusions from other laboratories, the present studies demonstrate modulation of the serotonin system during the day of proestrus. Changes in hypothalamic ^{3}H-5-HT binding during the day of proestrus were similar to those previously reported for frontal cortex. However, in contrast to frontal cortex, where the elevated proestrous ^{3}H-5-HT binding remained high for about 24 hours, hypothalamic binding was low by the afternoon of estrus. Since serotonin has been implicated both in the control of luteinizing hormone (LH) secretion (9, 11, 14, 17, 42) and in the regulation of sexual receptivity (2, 26–28) these proestrous changes in the serotonin system may be functionally significant for the female's transition from a receptive to a nonreceptive state.

Changing levels of estradiol probably contribute to estrous cycle modulation of ^{3}H-5-HT binding. In vivo treatment with estradiol decreased binding of 2–3 nM ^{3}H-5-HT binding in hypothalamic and cortical tissue and the present results suggest that estradiol alters the K_d rather than the B_{max} of serotonin binding sites. However, since the highest concentration of ^{3}H-5-HT used in these studies was 12 nM, it is possible that different results would be obtained for the lower affinity 5-HT binding sites. Since estradiol levels are highest on the morning of proestrus and decline during that afternoon and evening, we have suggested that the proestrous increase in cortical ^{3}H-5-HT binding reflected a withdrawal from the depressing effects of estradiol. The present time course for ^{3}H-5-HT binding changes after estradiol treatment of ovariectomized females does not support such a view. However, it should be noted that the injected estradiol exceeded physiological levels of the steroid so the time course may not faithfully represent the in vivo changes. Nevertheless, additional hormonal events are probably involved in the proestrous change in serotonin binding sites. An obvious candidate would be progesterone. Progesterone alone resembled estradiol in modulating ^{3}H-5-HT binding in ovariectomized rats (4), but when progesterone was administered following estradiol treatment, it attenuated the effects of estradiol. In addition, several investigators have suggested that the facilitatory effect of progesterone on sexual behavior is mediated through the serotonin system.

Proestrous changes in ^{3}H-5-HT binding are complex and appear to involve both alterations in K_d and B_{max}. Although the differences in intact females were not as large as those observed in the ovariectomized female following estradiol treatment, there was the suggestion of an increase in the K_d for ^{3}H-5-HT binding to hypothalami from females sacrificed on the night of proestrus. The major effect of estradiol may, therefore, be on the binding affinity and not on the number of serotonin receptors and the proestrous elevation of K_d may reflect this action.

The hypothalamus is a major target for circulating estradiol, so proestrous changes in the hypothalamic serotonergic system might be expected. However, since cortical tissue has few intracellular estradiol receptors, it is unlikely to be affected through such a mechanism. Nevertheless, Biegon and Samuel (5) and Biegon and McEwen (3) reported that reductions in ^{3}H-5-HT binding were obtained by in vitro incubation of forebrain tissue for two hours with estradiol (at much lower concentrations than used in the present study). These investigators compared the effects of estradiol to that of a PVP control and no effects of PVP were found when it was compared to a tris control. In contrast, we found PVP to have a significant effect on ^{3}H-5-HT binding but, in agreement with the previous investigations, estradiol attenuated this increase. Biegon and McEwen (3) suggested that estradiol had a direct effect on the microenvironment of the tissue. Although the mechanisms for this action were not elucidated, it was postulated that the steroid altered the fluidity of the membrane and, thereby, the accessibility of the ligand to the serotonin receptor. This hypothesis was derived from work reported by Heron *et al.* (13) showing that in vitro effects of cholesterol on serotonin binding were correlated with changes in membrane fluidity. However, the change in ^{3}H-5-HT binding after cholesterol was complex and not indicative of a simple alteration in the total number of binding sites. More importantly, in Heron *et al.*'s studies, both membrane fluidity and binding parameters were evaluated with a PVP control. Since PVP had distinct effects on the binding of ^{3}H-5-HT, it is difficult to evaluate the in vivo significance of PVP's attenuation by estradiol. Unfortunately, due to the low solubility of estradiol, any in vitro assessment of the effects of estradiol may be confounded by effects of the solubilizing agent.

In summary, these results demonstrate that estradiol can modulate ^{3}H-5-HT binding in tissue containing high densities of estradiol receptors and also in tissue with low concentration of the steroid receptor. Although variations in ^{3}H-5-HT binding are present in intact females during the estrous cycle, they are not faithfully represented by changing levels of estradiol alone. Other gonadal hormones (e.g., progesterone) may play a crucial role in the estrous cycle change in ^{3}H-5-HT binding sites.

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