Increase of Serotonin Receptors in Rat Uterus Induced by Estradiol*

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[3H]Spiroperidol was used to label uterine membrane-binding sites that have the characteristics expected of serotonergic receptors. The characteristics of specific [3H]spiropiperidol binding to the uterine membrane of 17β-estradiol-3-benzoate-treated ovariectomized rats were studied. The specific [3H]spiropiperidol binding was rapid and reversible, and the half-maximal saturation, taken as the apparent dissociation constant (Kd), for [3H]spiropiperidol, was 5.16 ± 0.24 (n = 12) nM [3H]spiropiperidol. Scatchard plots of saturation curves of the specific [3H]spiropiperidol binding were convex and the Hill coefficient was 2.06 ± 0.11 (n = 12). Cinanserin, mianserin, metergoline (which are serotonergic antagonists), and serotonin (5-HT) inhibited the [3H]spiropiperidol binding with apparent Kd values of 21.2, 14.1, 14.1, and 176.5 μM, respectively. Concentrations of 1 mM sulpiride (a dopaminergic antagonist) and dopamine reduced [3H]spiropiperidol binding only 26 and 23%, respectively. 1 mM GTP reduced the potency of 5-HT (10⁻¹⁰–10⁻³ M) to displace bound [3H]spiropiperidol. The uterine membranes were treated with various enzymes and protein-modifying reagents, and binding studies on the treated uterine membranes showed that protein(s), phospholipids, and N-acetylneuraminic acid in uterine membranes were important as specific binding sites of [3H]spiropiperidol.

Measurement of the specific binding of [3H]spiropiperidol to uterine membranes from untreated and estradiol-treated ovariectomized rats showed that estradiol significantly increased the number of specific binding sites of [3H]spiropiperidol, but did not change the apparent affinity of specific [3H]spiropiperidol binding. Estradiol also did not change the dissociation constant or the number of binding sites for [3H]3-quinuclidinyl benzilate, which binds to muscarinic acetylcholine receptors.

These findings suggest that [3H]spiropiperidol mainly binds to 5-HT receptors in the uterine membrane of estradiol-treated ovariectomized rats. The finding that administration of estradiol significantly increased the number of [3H]spiropiperidol-binding sites is consistent with the specific increase in the contractile response to 5-HT observed in isolated uterus from ovariectomized rats treated with estradiol.

In several species of mammals, the response of the uterus to contractile agents is dependent upon the sex steroid status of the animal (1–4). Erspamer (5) and Robson et al. (6) reported that the contractile response to 5-HT of ovariectomized rat uterus can be greatly increased by administration of estradiol, but not testosterone, progesterone, or other steroid hormones. Therefore, uteri isolated from ovariectomized rats after injection of estradiol have been widely used for bioassay of 5-HT (7). However, little is known about the mechanism(s) by which the contractile response to 5-HT in rat uterus is increased by administration of estradiol.

We recently confirmed that the contractile response of the uterus of ovariectomized rats to 5-HT was increased by administration of estradiol, and found that the effect of estradiol was specific for 5-HT, the contractile responses to ACh and oxytocin not being influenced by administration of estradiol. This specific increase in the contractile response to 5-HT seemed to be due to change in the number of 5-HT receptors, not to change in uptake or metabolic degradation of 5-HT.

It has been reported that in the cerebral cortex and hippocampus of rat brain the radioligand [3H]spiropiperidol mainly binds to 5-HT receptors, although in the corpus striatum of rat brain only a small portion of the [3H]spiropiperidol binds to 5-HT receptors, most of the radioligand binding to DA receptors (8–12). However, little is known about the characteristics of [3H]spiropiperidol binding to uterine membrane.

In this work, we therefore use the radioligand [3H]spiropiperidol to label binding sites with the characteristics expected for 5-HT receptors in uterine membranes, and investigate the characteristics of specific binding of [3H]spiropiperidol to the uterine membrane from estradiol-treated ovariectomized rat. Our major findings were that (a) the radioligand [3H]spiropiperidol mainly binds to 5-HT receptors, (b) the number of specific binding sites of [3H]spiropiperidol is significantly increased by administration of estradiol, without any change in the apparent affinity of specific binding of [3H]spiropiperidol, and (c) the administration of estradiol does not change the apparent dissociation constant or the number of binding sites for the radioligand [3H]QNB, which binds to muscarinic ACh receptors.

EXPERIMENTAL PROCEDURES

Virgin female Wistar rats weighing about 200 g were used. They were ovariectomized through a dorsal incision under ether anesthesia without regard to the stage of the estrous cycle. On day 15 after ovariectomy, they were given 10 μg of 17β-estradiol-3-benzoate once every 12 h for 48 h by intrasubcutaneous injection and then were killed. Untreated ovariectomized rats were used as controls.

Preparation of Uterine Membranes—Untreated and estradiol-treated ovariectomized rats were stunned by a blow on the head and the uterine horns were removed and placed in 50 mM Tris-HCl buffer (pH 7.4 at 30°C). The uterine horns in buffer solution were freed of connective tissue, washed, and homogenized in a Waring Blender. The homogenate was centrifuged at 105,000 × g for 30 min to remove nuclei and cell debris. The supernatant was ultracentrifuged at 100,000 × g for 60 min to remove microsomes and mitochondria. The supernatant was then centrifuged at 350,000 × g for 30 min. The supernatant contained the uterine membranes.

The abbreviations used are: 5-HT, serotonin; ACh, acetylcholine; GTP, guanosine 5'-triphosphate; QNB, 3-quinuclidinyl benzilate.

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from fat and loosely bound connective tissue and homogenized in 70 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 30 °C) using a Physcotron (Niti-on Co.; setting 60–70) for 20 s. The homogenate was filtered through nylon mesh and then centrifuged at 42,000 × g for 20 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4 at 30 °C) and recently centrifuged at 42,000 × g for 20 min. The pellet was suspended in 10 mM Tris-HCl buffer (pH 7.4 at 30 °C) at 2–3 mg of protein/ml (uteri from estradiol-treated ovariectomized rats) or 0.5–2 mg of protein/ml (uteri from untreated ovariectomized rats) and placed in an ice bath until used.

Binding Assay of Radiolabeled Ligands—For the binding assay of [3H]spiroperidol, a 100-μl aliquot (50–300 μg of protein) of a suspension of uterine membranes was added to assay medium consisting of 62.5 mM Tris-HCl (pH 7.4 at 30 °C) and 0.25 mg/ml L-ascorbic acid with various concentrations (2.5–17.5 nM) of [3H]spiroperidol. The final volume of the assay system was 500 μl. After incubation for 5 min at 30 °C, unless otherwise indicated, the mixture was rapidly filtered through a Whatman GF/F glass filter under vacuum and the filter was washed four times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 30 °C). The filter was transferred to a counting vial, 5 ml of Triton/toluene-based scintillation mixture was added, and the radioactivity was counted in a liquid scintillation counter (Packard Model 3380). All assays were performed in duplicate or triplicate.

RESULTS

Properties of [3H]Spiroperidol Binding to Uterine Membranes of Estradiol-treated Ovariectomized Rats—The specific binding of [3H]spiropemidol was saturable and reached a plateau at about 10 nM (Fig. 4A). Half-maximal saturation, taken as the apparent dissociation constant (Kp) for the interaction of [3H]spiropemidol with binding sites, occurred with 5.16 ± 3.81 nM.

From the data in Fig. 4B, the equilibrium dissociation constant (Kd) was calculated from Hill plots (16). Hill coefficients of specific [3H]spiropemidol binding to untreated and estradiol-treated preparations were obtained from Hill plots (16). Kd values were calculated from IC50 values (reagent concentration inhibiting 50% of specific binding) according to the equation of Cheng and Prusoff (17).

Statistical analyses were made by Student’s t test and a level of p < 0.05 was regarded as significant.

Reagents—17β-Estradiol-3-benzoate was purchased from Teikoku Co. Guanosine 5′-triphosphate was from Yamasa Shoyu Co. Acetylcholine, N-bromosuccinimide, 5,5′-dithiobis(2-nitrobenzoic acid), dopamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, N-ethylmaleimide, 2-hydroxy-5-nitrobenzyl bromide, and serotonin were from Wako Co. Atropine, α-chymotrypsin, oxytocin, phospholipase A, and trypsin were from Wako Co. Cinanserin was a gift from Squibb (S. J. Lucania), metergoline was from Dr. K. Saito (Osaka University, School of Dentistry), mianserin was from Organon Co. (Japan; H. Mita), spiroperidol was from Janssen Pharmaceutica (Belgium; Dr. P. Laduron) and Esai Co. (Japan) and sulpiride was from Fujisawa Yakuhin Co. (Japan; Dr. T. Nuki).

All drugs were prepared in distilled water or 0.01 N HCl solution on the day of use and were neutralized when necessary before use.

![Fig. 1. Kinetics of specific binding of [3H]spiropemidol to uterine membranes of estradiol-treated ovariectomized rats. A, specific [3H]spiropemidol binding as a function of time. [3H]spiropemidol (2 nM) was incubated with uterine membranes (200–300 μg of protein/tube) for the indicated times at 30 °C and specific binding was determined as described under "Experimental Procedures." Points are means for three or four separate animals. Bars indicate standard errors. B, reversibility of specific [3H]spiropemidol binding. Membranes were incubated with [3H]spiropemidol (2 nM) for 5 min at 30 °C and then a large excess of unlabelled spiropemidol (10 μM) was added. The time of spiropemidol addition is defined here as t = 0. At the indicated times subsequently, specific [3H]spiropemidol binding was determined as described under "Experimental Procedures." Points are means for five to seven separate animals. Bars indicate standard errors.](http://www.jbc.org/content/journal/jbc/198/21/13439/F1.big)
treated uterine membranes with various enzymes and protein-modifying reagents and then measured their binding sites (Table I). [3H]Spiroperidol binding was not reduced by treatment with low concentrations of trypsin (3.8 units/ml) or α-chymotrypsin (0.43 unit/ml), but was significantly reduced by higher concentrations of trypsin (1140 units/ml) and α-chymotrypsin (43 units/ml). These findings suggest that the binding sites of [3H]spiropenrol involve protein structure. Pretreatments with phospholipase A2, D, and neuraminidase significantly reduced the specific binding of [3H]spiropenrol. The finding that pretreatment with neuraminidase reduced the specific binding of [3H]spiropenrol is consistent with the observation that the contractile response of isolated smooth muscle to 5-HT was inhibited selectively by treatment of neuraminidase plus EDTA (18). These findings suggest that phospholipids and N-acetylneuraminic acid in uterine membranes are directly or indirectly important for specific binding sites of [3H]spiropenrol. Specific [3H]spiropenrol binding

0.24 (n = 12) nM [3H]spiropenrol. The amount of [3H]spiropenrol specifically bound at equilibrium was 1.23 ± 0.11 (n = 12) pmol/mg of protein. The specific binding accounted for about 70% of the total binding at a concentration of 2 nM, the amount of [3H]spiropenrol used routinely in various other studies (data not shown). With an increase in the ligand concentration above 6 nM, the percentage of total binding attributable to specific binding decreased and with 14 nM [3H]spiropenrol it was 51% of the total binding (data not shown). Nonspecific binding increased linearly with an increase in [3H]spiropenrol concentration (data not shown).

The specific binding of [3H]spiropenrol was time-dependent (Fig. 1A). At 30 °C, the specific binding reached a plateau after 5 to 16 min, and was half-maximal after about 2 min. The specific binding of [3H]spiropenrol was also reversible (Fig. 1B).

The competitions of various reagents for [3H]spiropenrol-binding sites were investigated (Fig. 2). The [3H]spiropenrol binding was inhibited by cinanserin, mianserin, metergoline, and 5-HT, which caused half-maximal inhibitions at about 30, 20, 20, and 250 μM, respectively. Therefore, the apparent K values for cinanserin, mianserin, metergoline, and 5-HT are about 21.4, 14.1, 14.1, and 176.5 μM, respectively. Cinanserin, mianserin, and metergoline are known to be 5-HT antagonists. On the other hand, at concentrations of 1 nM, dopamine and sulpiride (which affect dopamine receptors) reduced [3H]spiropenrol binding by only 26 and 23%, respectively. These findings suggest that the radioligand [3H]spiropenrol mainly binds to 5-HT receptors in uterine membranes of estradiol-treated ovariectomized rats.

Fig. 3 shows the effect of 1 mM GTP on displacement of bound [3H]spiropenrol by 5-HT in uterine membranes of estradiol-treated ovariectomized rats. Membranes were incubated with [3H]spiropenrol (2 nM) in the absence and presence of 5-HT and/or GTP, and specific binding was determined as described under "Experimental Procedures." Points are means ± S.E. for three to five separate animals. *p < 0.05; **, p < 0.02; significance of difference from percentage inhibition produced with 5-HT alone.

![Table I](image)

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific [3H]spiropenrol binding</th>
<th>p</th>
<th>n</th>
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<td>Enzymes</td>
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<tr>
<td>α-Chymotrypsin, 0.43 unit/ml</td>
<td>96.8 ± 35.8</td>
<td>NS*</td>
<td>3</td>
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<tr>
<td>Trypsin, 3.8 units/ml</td>
<td>47.5 ± 8.9</td>
<td>&lt;0.02</td>
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<tr>
<td>5-HT 1140 units/ml</td>
<td>54.9 ± 5.2</td>
<td>&lt;0.05</td>
<td>3</td>
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<tr>
<td>Neuraminidase, 0.03 unit/ml</td>
<td>65.1 ± 10.3</td>
<td>&lt;0.02</td>
<td>5</td>
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<tr>
<td>Phospholipase A2, 1.3 units/ml</td>
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<td>&lt;0.001</td>
<td>4</td>
</tr>
<tr>
<td>Phospholipase D, 2.0 units/ml</td>
<td>47.5 ± 7.4</td>
<td>&lt;0.01</td>
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<td>Protein-modifying reagents</td>
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<tr>
<td>N-Bromosuccinimide, 10 μM</td>
<td>63.6 ± 7.7</td>
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<td>3</td>
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<td>5,5'-Dithiobis(2-nitrobenzoic acid), 5 mM</td>
<td>75.0 ± 16.7</td>
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<td>3</td>
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<tr>
<td>N-Ethylmaleimide, 1 mM</td>
<td>53.2 ± 5.9</td>
<td>&lt;0.05</td>
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<tr>
<td>1-Ethyl-5-(3-dimethylaminopropyl)carbodiimide, 1 mM</td>
<td>40.9 ± 5.4</td>
<td>&lt;0.001</td>
<td>6</td>
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<tr>
<td>2-Hydroxy-5-nitrobenzyl bromide, 10 mM</td>
<td>126.5 ± 8.6</td>
<td>NS</td>
<td>3</td>
</tr>
</tbody>
</table>

*NS, not significant.

![Fig. 2](image)

**Fig. 2. Inhibition of [3H]spiropenrol binding by various agents.** Uterine membranes of estradiol-treated ovariectomized rats were incubated with [3H]spiropenrol (2 nM) in the absence and presence of the indicated agents, and specific binding was determined as described under "Experimental Procedures." Points are means for four to six separate animals. Sul, sulpiride; DA, dopamine; Met, metergoline; Cin, cinanserin; Spi, spiropenrol; Mia, mianserin.

![Fig. 3](image)

**Fig. 3. Effect of GTP on the displacement of bound [3H]spiropenrol by 5-HT in uterine membranes of estradiol-treated ovariectomized rats.** Membranes were incubated with [3H]spiropenrol (2 nM) in the absence and presence of 5-HT and/or GTP, and specific binding was determined as described under "Experimental Procedures." Points are means ± S.E. for three to five separate animals. *p < 0.05; **, p < 0.02; significance of difference from percentage inhibition produced with 5-HT alone.
was sensitive to reagents which modify sulfhydryl groups. It was reduced markedly and similarly by pretreatments with 10 μM N-bromosuccinimide and 1 mM N-ethylmaleimide, and somewhat less by treatment with 5 mM 5,5'-dithiobis(2-nitrobenzoic acid). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1 mM), a carboxyl group reagent, reduced specific [3H]spiroperidol binding about 59%. Pretreatment with 10 mM 2-hydroxy-5-nitrobenzyl bromide, which alters tryptophan residues, did not reduce specific [3H]spiroperidol binding. These findings suggest that sulfhydryl and carboxyl groups in uterine membranes are important to the specificity of the binding sites of [3H]spiroperidol.

Effect of Estradiol Administration on Specific [3H]Spiroperidol Binding to Uterine Membranes—Previously, we found that the contractile response of ovariectomized rat uterus to 5-HT was specifically increased by administration of estradiol; the contractile responses to ACh and oxytocin were not influenced by its administration.2 Therefore, we measured 5-HT receptors quantitatively by determining [3H]spiroperidol binding to the uterine membrane of untreated and estradiol-treated ovariectomized rats. In both preparations, specific binding of [3H]spiroperidol was saturated at a concentration of about 10 nM, and the concentrations for half-maximal saturation were 5.22 ± 0.22 (n = 11) and 5.16 ± 0.24 (n = 12), nM, respectively (Fig. 4A). However, the amount of [3H]spiroperidol specifically bound at equilibrium was significantly increased by administration of estradiol; that is, it was 0.56 ± 0.04 (n = 11) pmol/mg of protein in untreated preparations and 1.23 ± 0.11 (n = 12) pmol/mg of protein in estradiol-treated preparations. Scatchard analysis of saturation curves of specific [3H]spiroperidol binding to untreated and estradiol-treated preparations gave convex curves (Fig. 4B) and the Hill coefficients were 2.10 ± 0.14 (n = 11) and 2.06 ± 0.11 (n = 12), respectively (Fig. 4C), indicating that specific [3H]spiroperidol binding to untreated and estradiol-treated preparations shows positive cooperativity. ACh receptors were also measured quantitatively with [3H]QNB under conditions similar to those for [3H]spiroperidol binding (Fig. 5, A and B). The pKD value and the number of binding sites for [3H]QNB were not significantly changed by administration of estradiol (pKD for [3H]QNB, 0.43 nM for untreated and 0.33 nM for estradiol-treated preparations; number of binding sites for [3H]QNB, 126 fmol/mg of protein for control and

**FIG. 4.** Equilibrium binding of [3H]spiroperidol to uterine membranes of untreated and estradiol-treated ovariectomized rats. A, specific [3H]spiroperidol binding as a function of the concentration of [3H]spiroperidol. [3H]spiroperidol at the indicated concentrations was incubated with uterine membranes of untreated and estradiol-treated ovariectomized rats for 5 min at 30 °C and specific binding was determined as described under “Experimental Procedures.” B, data shown as a Scatchard plot. C, data shown as a Hill plot. Points are means for 16 to 12 separate animals. Bars indicate standard errors. *p < 0.05; **p < 0.02; significance of difference from [3H]spiroperidol bound to uterine membranes of untreated rats.

108 fmol/mg of protein for estradiol-treated preparations). These findings suggest that the amount of 5-HT receptor was significantly increased by administration of estradiol, without any change in the apparent affinity of 5-HT receptor to 5-HT and that the amount and the apparent affinity of muscarinic ACh receptor did not change after administration of estradiol. Therefore, these findings are consistent with the observation that the contractile response to 5-HT, but not to ACh or oxytocin, was increased by administration of estradiol.2

**FIG. 5.** Equilibrium binding of [3H]QNB to uterine membranes of untreated and estradiol-treated ovariectomized rats. A, specific [3H]QNB binding as a function of the concentration of [3H]QNB. Experiments were performed as described in Fig. 4B; data are shown as a Scatchard plot. Points are means for four to six separate animals. Bars indicate standard errors.

**DISCUSSION**

The contractile response to 5-HT of isolated uterus was specifically increased by administration of estradiol (5, 6). The specific increase in sensitivity to 5-HT could be due to (a) change in metabolic degradation of 5-HT, (b) change in 5-HT uptake, or (c) change in 5-HT receptors. Our results suggested that it was due to change in the 5-HT receptor, not to change in uptake or metabolic degradation of 5-HT. In studies on the role of changes in 5-HT receptors in the increase of serotonergic sensitivity upon administration of
estradiol, we first used the radioligand [3H]5-HT in binding studies. But we found that [3H]5-HT binding to rat uterine membranes did not satisfy the so-called "basic criteria" (19) for a receptor, although it has been reported that [3H]5-HT labels 5-HT receptors in the central nervous system (20, 21); that is, specific binding of [3H]5-HT (at concentrations of 1, 10, and 100 nM) were not reversible and were not inhibited by 5-HT antagonists (mecamylamine, methysergide, and ergometrine) at high concentrations such as $10^{-4}$--$10^{-3}$ M, although the specific binding of [3H]5-HT was saturable. Therefore, we used the radioligand [3H]spiroperidol to label binding sites with the characteristics expected for 5-HT receptors. Specific [3H]spiroperidol binding appears to satisfy the criteria for identification of 5-HT receptors. Indeed, the specific binding of [3H]spiroperidol was rapid, reversible, and saturable (Figs. 1A, 1B, and 4A). Moreover, cinanserin, metgepamine, mianserin, and 5-HT inhibited the binding of [3H]spiroperidol (Fig. 2). We also found that cinanserin, metgepamine, mianserin, and spiroperidol specifically inhibited the contractile response to 5-HT in isolated uterus from estradiol-treated ovariectomized rats, because at concentrations that completely inhibited the contractile response to 5-HT they did not affect the contractile response to ACh or oxytocin. However, this possibility seems unlikely because the amounts of specifically bound [3H]spiroperidol (at concentrations of 0.4-18 nM [3H] spiroperidol) in untreated and estradiol-treated preparations did not change irrespective of whether L-ascorbic acid (0.25 mg/ml) was present in the medium (data not shown).

We found that isolated uterus the maximal contractile response to 5-HT, but not ACh or oxytocin, was significantly greater in estrus than in diestrus.2 The estradiol level of rats is highest in proestrus and is still elevated during estrus (29, 30). Thus, the contractile response to 5-HT may be sensitive to changes in the level of sex steroid hormone during the estrous cycle, and the specific change in sensitivity to 5-HT may be important physiologically in some uterus function(s). However, the functional role of 5-HT receptors in the uterus and the physiological significance of their increase by estrogen are unknown. There are also reports that estrogen modulates the receptors for hormones and neurotransmitters in several systems of myometrium (31-35).

Although the mechanism(s) of the increase in number of 5-HT receptors on administration of estradiol is unknown, we consider that a change in 5-HT content of the uterus after administration of estradiol may be correlated with an increase in the number of 5-HT receptors upon administration of estradiol, because it has been reported that a decrease of the 5-HT content of the uterus of estradiol-treated ovariectomized rats occurred without apparent degradation of mast cells (36). Further investigations are needed on the mechanism(s) of increase of 5-HT receptors after administration of estradiol.

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4. Ichida, manuscript in preparation.
Increase of serotonin receptors in rat uterus induced by estradiol.
S Ichida, H Tokunaga, Y Oda, N Fujita, A Hirata and T Hata


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