**Endocrine,** *vol. 15, no. 2, 187–192, July 2001* 0969–711X/01/15:187–192/$11.50 

# Release of Norepinephrine from Human Ovary

## *Coupling to Steroidogenic Response*

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**We investigated the possibility that norepinephrine from the human ovary is released after nerve stimulation and that this neurotransmitter is coupled to a steroidogenic response. Biologically significant levels of both norepinephrine and dopamine were found in human ovarian biopsies. [$^3$H]norepinephrine incorporated in vitro was readily released by electrical stimulation in a $Ca^{2+}$-dependent process. Ovarian membrane preparations exhibited specific binding sites for the β-adrenergic antagonist [$^3$H]dihydroalprenolol. Displacement of [$^3$H]dihydroalprenolol with zinterol (a specific $\beta_2$-agonist) indicated that 72% of these sites were type $\beta_2$-receptors. β-receptors were also present on granulosa cells. Stimulation of granulosa cells with luteinizing hormone or the β-agonist isoproterenol increased the release of progesterone after 4 d in culture. These results suggest that the sympathetic nerves present in human ovary are coupled to β-adrenergic receptors present in endocrine cells and, as in nonprimate mammals, appear to participate in the regulation of ovarian function.**

**Key Words:** Sympathetic nerves; β-receptor, human ovary.

### Introduction

The mammalian ovary has a rich sympathetic nerve supply with norepinephrine as the major sympathetic neurotransmitter *(1)*. Histochemical studies of catecholamine fluorescence in the human ovary have revealed a network of fluorescent fibers penetrating the ovary through the hilar region and distributed through the perivascular region, the stroma, and the follicular thecal layers *(2,3)*. A role for catecholamines in ovarian physiology in nonprimate mammals has been suggested by the effects of this neurotransmitter on progesterone and androgen secretion (for a review see ref. *4*), acquisition of follicle-stimulating hormone receptor and aromatase enzyme in immature rat granulosa cells *(5)*, and development of polycystic ovary in the rat *(6)*. Nerve terminals in the human ovary are, as in other mammals, in close synaptic contact with the thecal layer, but they do not appear to cross the follicular basement membrane into the granulosa cell layer (for a review, see ref. *7*). No further functional characterization of the nerves has been published, although there have been many studies on the putative role of catecholamines in ovarian physiology, particularly in the contraction and relaxation of the follicular wall *(8,9)*.

A role for catecholamines in regulation of progesterone release from human granulosa cells has, however, been more difficult to demonstrate. Although norepinephrine stimulates progesterone release from thecal and granulosa cells in the nonprimate ovary (for a review, see ref. *4*), there are divergent opinions about the role of catecholamines in steroid secretion from the primate ovary. In some studies, norepinephrine and/or β-agonists appear to stimulate *(10)*, potentiate the effect of gonadotropin *(11)*, or have no effect *(12)* on progesterone secretion by granulosa cells.

A physiologic role for endogenous catecholamines in ovarian function would be supported by a relationship between norepinephrine released at sympathetic terminals and its action on postsynaptic receptors present in endocrine cells. Such a relationship would be distinct from the effects of norepinephrine attributable to the action of plasma catecholamines. We have employed neurochemical techniques to demonstrate the existence of functionally active noradrenergic nerve terminals in the human ovary. We have also characterized β-adrenergic receptors and suggest that these are related to the adrenergic-mediated secretory response.

Received March 19, 2001; Revised May 30, 2001; Accepted May 30, 2001.
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## Results

### *Concentration of Norepinephrine, Dopamine, and Epinephrine in Ovary*

As we can expect from a tissue innervated by sympathetic nerves, the human ovary presented a high content of norepinephrine (1.23 ± 0.07 μg/g of ovary, $n = 5$ mean ± SEM) and readily detected amounts of dopamine (0.36 ± 0.04 μg/g of ovary, $n = 5$ mean ± SEM). Epinephrine could not be detected (lower than 10 pg/mg).

### *Release of Incorporated Norepinephrine*

Incubation of neural tissue with norepinephrine results in incorporation of norepinephrine through a transmembrane carrier *(13)*, specific to neural tissue *(14)*. Incubation of ovarian tissue (10 mg) with [$^3$H]norepinephrine incorporated a significant fraction (4.5 ± 0.5%, $n = 3$, mean ± SEM) of the total radioactivity of the incubation medium *(15)*. Transmural stimulation of the ovary (arrows in Fig. 1) at the frequency of the normal firing rate of the peripheral sympathetic nerves produced a 10-fold increase in the release of [$^3$H]norepinephrine into the superfusion medium (5.4 ± 0.25 vs 0.55 ± 0.04% spontaneous release) (Fig. 1A, left) and easily responded to a second period of stimulation (Fig. 1A, right). Removal of $Ca^{2+}$ from the medium and addition of 0.1 m*M* EGTA abolished the release of norepinephrine (Fig. 1B, right).

### *Binding of [$^3$H]Dihydroalprenolol to Ovarian Membranes*

Ovarian membranes were incubated at 37ºC for 30 min with 0.5–4.0 n*M* dihydroalprenolol (DHA, β-antagonist) (Fig. 2). A maximal specific binding of 114 fmol of DHA/mg of protein was found. Scatchard analysis revealed a single binding site with $K_d = 3.3$ n*M*. Experiments involving competition between DHA (2 n*M*) and propranolol resulted in a total displacement of the binding with an $IC_{50}$ of 2.5 n*M* (Fig. 3). When the $\beta_1$-antagonist nadolol replaced propranolol, the maximal displacement was 30% of that obtained with propranolol and the $IC_{50}$ was 16 n*M*. Zinterol (a $\beta_2$-agonist) decreased DHA binding by 72% with an $IC_{50}$ of 1 n*M*.

Because the ovarian membrane preparation principally contains interstitial tissue, thecal interstitial cells, and stroma, we also prepared membranes from granulosa cells after 4 d of incubation. Incubation of this preparation with a saturating concentration of DHA (10 n*M*) showed specific binding that was displaced by propranolol. The maximal specific binding/mg of protein was 3.20 ± 1.00 pmol/mg of protein ($n = 3$ mean ± SEM), a value higher than that obtained with the ovarian membrane preparation. This finding represents a threefold increase as compared with β-adrenergic binding after 24-h of incubation.

### *β-Adrenergic-Induced Secretion of Progesterone from Granulosa Cells*

As a result of the aforementioned observations, we tested the ability of isoproterenol to induce the secretion of progesterone from granulosa cells. As shown in Fig. 4, neither luteinizing hormone (LH) nor isoproterenol increased progesterone production during a 24-h incubation of granulosa cells. Both human chorionic gonadotropin (hCG) and isoproterenol, however, did stimulate progesterone production in incubations in culture medium for 4 d.

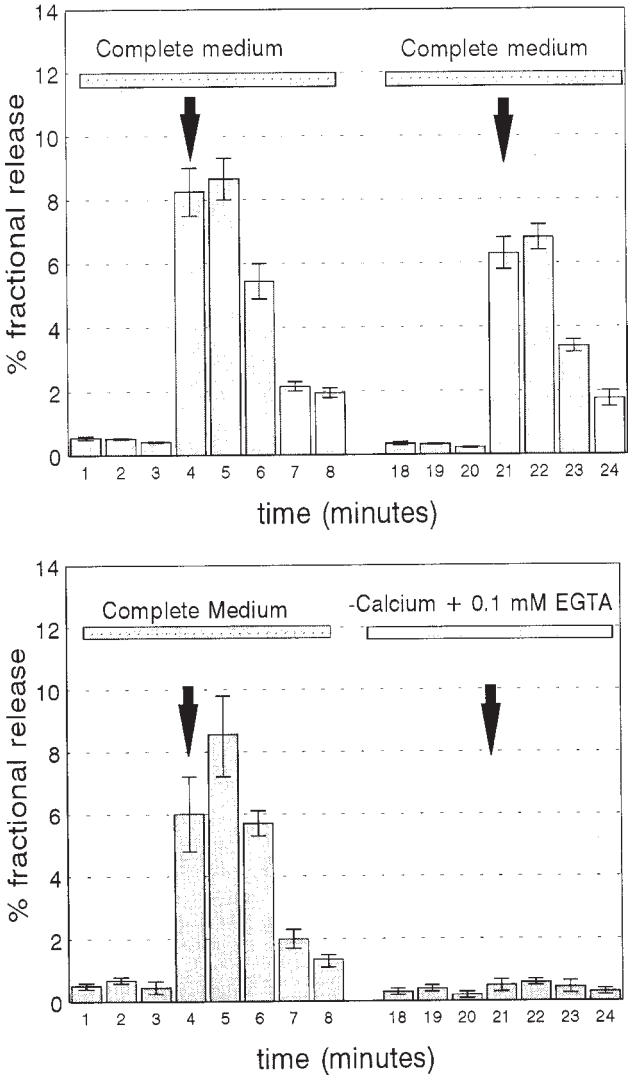


**Fig. 1.** Release of [$^3$H]norepinephrine from the human ovary and its dependence on extracellular calcium. A piece (15 mg) of an ovarian biopsy was preincubated with [$^3$H]norepinephrine and then stimulated with a train of 600 pulses at 10 Hz, 2-ms length, and 80 V for 1 min (arrows). Four minutes after the first stimulation, the perifusion medium was replaced with Krebs-bicarbonate buffer (complete medium), without calcium and with 0.1 m*M* EGTA, and the tissue was stimulated as described above. (**A**) Response of the ovary when two stimulation periods were applied. (**B**) Response of the ovary when calcium was eliminated during the second period of stimulation. Results are expressed as a percentage of the fractional release and are means ± SEM from five patients.

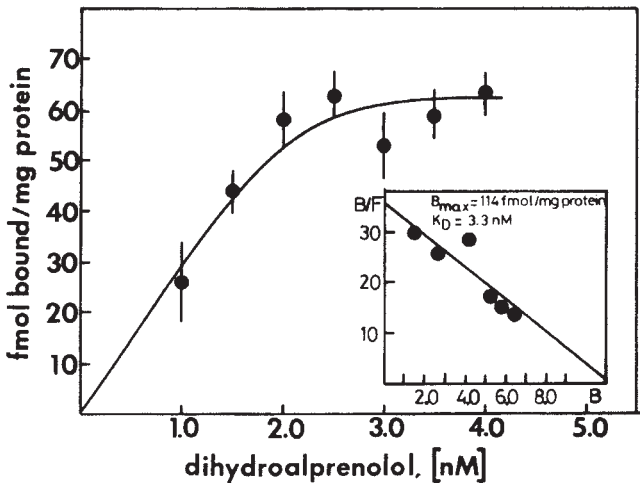


**Fig. 2.** Binding of [$^3$H]dihydroalprenolol to human ovary membranes. Membranes (70 µg protein) were incubated at 37ºC for 30 min as described in the text. Values are means ± SEM of five experiments, each one performed in duplicate. The insert shows a Scatchard plot of the data.

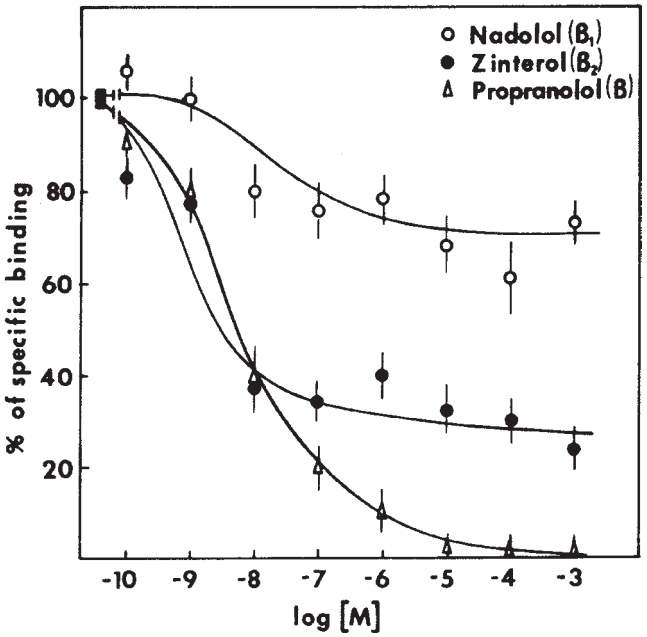


**Fig. 3.** Displacement of [$^3$H]dihydroalprenolol (10 n*M*) specifically bound to a membrane fraction obtained from the ovary. Incubation was for 30 min at 37ºC with DL-propranolol (△), nadolol (○), and zinterol (●). Results are means ± SEM of five experiments performed in duplicate.

## Discussion

Our results suggest that the human ovary has a functional sympathetic innervation coupled to steroid secretion. The value for the concentration of norepinephrine in the ovary was similar to others previously reported and 10 times higher than rat ovarian tissue *(2,15–17)*. Data on dopamine concentration in ovarian follicular fluid from human follicle have been reported *(18)*, but no data on dopamine concentration in the ovarian tissue from humans have been reported. Tissue concentration of dopamine determined in the present study is, however, higher than expected if dopamine were acting only as a precursor of norepinephrine. The concentration found (2.5 µ*M*) is sufficient to activate any known dopaminergic receptors that might be present in the ovary. Mayerhofer et al. *(19)* recently described the presence of $D_1$-dopaminergic receptor in the human ovary. Although they did not speculate as to a role for this putative receptor, it could participate in the ovarian physiology through activation by locally released dopamine. This may be important because one treatment for anovulation resulting from an increased secretion of prolactin is the administration of the dopaminergic agonist, bromocriptine. Because the administration of bromocriptine to patients with normoprolactinemic amenorrhea increases their rate of ovulation *(20)*, it suggests that this compound might act at the ovarian as well as the central level. The overall concentrations of both catecholamines are at least 100 times higher than those in plasma from normal subjects *(21)*, suggesting a neuronal origin for both norepinephrine and dopamine. The fact that epinephrine was not detected at the ovarian tissue, where the existence of phenylethanol amine-*N*-methyl transferase (PNMT, the enzyme that catalyzes the transformation of norepinephrine to epinephrine) has not been documented, reinforces the idea that there is no contamination with plasma catecholamines; thus, the detected levels of norepinephrine and dopamine could represent catecholamines from locally synthesized neurotransmitter from sympathetic nerve terminals but not plasma catecholamines.

Are ovarian catecholamines functionally associated with sympathetic nerves and can they be released when the nerves are stimulated? The technique of electrical stimulation of ovarian tissue containing preloaded catecholamine has been shown to be valid in a variety of tissues and animal species *(22,23)* and permits analysis of nerve activity in tissue biopsies. Because of the participation of extracellular calcium in the neurotransmitter's storage vesicle recruitment and fusion to the cytoplasmic membrane to release neurotransmitter by exocytosis *(23)*, a basic criterion for establishing the neuronal origin of released [$^3$H]norepinephrine is an absolute dependence on extracellular $Ca^{2+}$, as was shown in the present study for human ovary and previously for other species *(15,16)*.

This technical approach may be useful in studies of neuronal activity in the diseased ovary such as the polycystic ovary syndrome (PCOS). Semenova *(24)* showed an increase in catecholamine fluorescence in the ovaries of women with PCOS but, because norepinephrine released to the synaptic cleft is the neurotransmitter available to act on postsynaptic receptors, an increase in the tissue concentration of the neurotransmitter is not enough to suggest an increased neuronal activity of the tissue. In support of this, we have

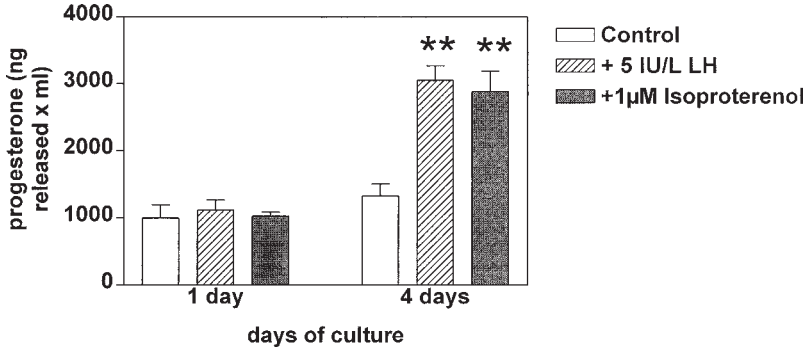


**Fig. 4.** Effect of isoproterenol and LH on progesterone secretion from human granulosa cells. After cells were obtained and isolated from red cells, 200,000 cells per well were incubated in 1:1 Dulbecco's modified Eagle's medium (DMEM):Ham's F12 (containing 10% human serum) alone (control) or with 5 IU/liter of LH or $10^{-6}$ *M* DL-isoproterenol for 24 h (short-term incubation, 1 d) or 4 d (change of medium every 24 h); progesterone released into the medium was determined by radioimmunoassay (RIA). For the 4-d incubation, results indicate progesterone released during the last 24-h period of incubation. Results are means ± SEM from 10 patients.

found that a stress-induced increase in ovarian nerve activity in the rat is not accompanied by an increase in norepinephrine concentration at the ovary but of a clear increase in the release of norepinephrine *(25)*. In other situations, however, we have found a clear correlation between changes in norepinephrine content and release, as the increase in norepinephrine content and release of the ovary of rats treated with estradiol valerate to induce PCO *(6,25)*, giving further support to the importance of the release of neurotransmitter as an index of neuronal activity instead of only content.

The level of secretory activity of ovarian sympathetic nerves is indicated by the substantial proportion of [$^3$H]norepinephrine released by electrical stimulation. If we assume an even distribution of radioactive norepinephrine in the total pool of norepinephrine in the tissue, we can calculate that 10 ng of norepinephrine is released locally into the synaptic cleft—a concentration of 0.01 μ*M* for the ovary as a whole. This value is close to the $K_d$ we found for the β-receptor, indicating that both norepinephrine and β-receptor sites may be in close proximity within the human ovary. We have not found data about pharmacologic characterization of β-adrenergic binding sites in the human ovary, but our data compared well with data described for the rat ovary *(26)*. The data described in the present study suggest that human ovarian secretory cells also respond to β-adrenergic agonist. The difference in the response of fresh and cultured granulosa cells to LH and isoproterenol may reflect the metabolic state of the cells or a different degree of luteinization occurring during culture *(10)*. Furthermore, because almost all in vitro fertilization procedures used similar hormonal treatments, it could explain the discrepancy in the literature regarding the effects of norepinephrine or agonists and, more recently, the lack of effect of dopamine on progesterone secretion from granulosa cells derived from in vitro fertilization procedures *(19)*. Our data confirmed the previous observation *(10)* that granulosa cells (obtained from in vitro fertilization procedures), only respond to gonadotropins and β-adrenergic agonists after at least 4 d of culture. Although both human and monkey ovary present a well-developed network of sympathetic nerves, data show differences between human and monkey ovary regarding the functional response to sympathetic neurotransmitters. The adenylate cyclase system of macaque corpus luteum was not responsive to catecholamines *(27)*; thus, monkey ovary may lack a potential neural mechanism for control of luteal function that is available to human ovary, which might explain differential responses to catecholamines, including the failure of dopamine to modify basal and hCG-stimulated progesterone secretion from isolated luteal cells *(28)*.

## Conclusion

Our data strongly suggest that sympathetic nerves from the human ovary release norepinephrine after stimulation and that released norepinephrine is coupled to steroid production from the ovary acting on $\beta_2$-adrenergic binding sites. This suggests a physiologic role for the sympathetic innervation of the human ovary and proposes a novel means for studying the function of this innervation in health and disease.

## Materials and Methods

### *Ovarian Biopsies*

Eight biopsy samples of human ovaries (each approx 100 mg) were obtained from women 42–46 yr of age with regular 26- to 32-d ovulatory cycles who were undergoing surgery for unrelated conditions. Patients, registered at Hospital del Salvador, Hospital San Jose, or Hospital J J Aguirre (Santiago), and with full obstetric histories, were informed of the study and gave their written consent according to institutional regulation. Laparoscopic surgery accompanied by surgical obtention of tissue was performed during the follicular phase (d 5 and 10 of the menstrual cycle).

### *Isolation of Granulosa Cells*

Human granulosa cells were isolated from follicular fluid obtained from multiple preovulatory follicles of women undergoing assisted in vitro fertilization. The cells used in this study were from 10 patients from the Clinica Alemana (Santiago). The patients were treated with standard hormonal regimes prior to follicular puncture (stimulation with leuprolide acetate; monitoring of follicular growth by ultrasound; and daily monitoring of estradiol, progesterone, and LH). Ovulation was stimulated by hCG (10,000 IU) when follicles reached a diameter of 20 mm. Follicular aspiration (guided by sonography) was performed 32–36 h after induction of ovulation in all women with a continuous rise in serum estradiol.

### *Culture of Granulosa Cells*

Granulosa cells were recovered from follicular fluid after centrifuging at 250*g* for 10 min. Cells from a single patient were suspended in 20–30 mL of phosphate-buffered saline, filtered through a sterile nylon mesh to remove any clumps, and separated from erythrocytes on Percoll (Pharmacia, Uppsala, Sweden). Cell suspensions were used for short- (1 d) or long-term (4 d) incubation in basal conditions or in the presence of $10^{-6}$ *M* isoproterenol or 5 IU/liter of LH. For both protocols, cells obtained from each patient were cultured separately and placed in triplicate in wells containing 200,000 cells and with a final volume of 1 mL with 1:1 DMEM and Ham's F12 containing 10% human serum. Serum was heat inactivated by warming at 56ºC for 30 min and sterilized by passing through a membrane filter (0.2 μm). In long-term incubations, media were changed every 24 h including LH or isoproterenol depending experimental conditions; medium was stored at –20ºC. Progesterone released to the medium was measured by RIA *(29)*.

### *Measurement of Norepinephrine, Dopamine, Epinephrine, and Protein*

Samples of ovarian tissue (10 mg) were homogenized in 10 vol of 0.2 *M* $HClO_4$. Suspensions were centrifuged at 15,000*g* for 10 min. norepinephrine and dopamine present in the supernates were determined by the method of Saller and Zigmond (30), as previously described (16).

### *Release of Norepinephrine from Ovary*

The procedure was previously described (16) with minor modifications. Each piece of ovarian tissue (15 mg) was preincubated for 20 min in Krebs-bicarbonate buffer (pH 7.4), gassed with 95% $O_2$:5% $CO_2$, and then incubated for 30 min at 37ºC with 2 μCi of [$^3$H]norepinephrine (40.1 Ci/mmol; Dupont/NEN, Boston, MA). Radioactivity not retained by the tissue was removed by further incubation for 60 min in Krebs-bicarbonate free of [$^3$H]norepinephrine. To study the spontaneous and stimulated release of [$^3$H]norepinephrine, the tissue was transferred to a thermoregulated perifusion chamber (1-mL capacity) provided with two platinum electrodes located in parallel at a distance of 7 mm from each other.

The perifusion fluid was Krebs-bicarbonate and the flow rate was 2.5 mL/min. Fractions were collected at 1-min intervals. After 3 min of collection, a train of 600 monophasic pulses (80 V, 10 Hz, 2 ms) was applied using a Grass S-4 stimulator; fractions were collected for 5 min. To study the extracellular $Ca^{+2}$ dependence of the release of norepinephrine, Krebs-bicarbonate was changed by the same buffer but without calcium plus 0.1 m*M* EGTA, the perifusion continued for 10 min, and the ovary was subjected to a second stimulation period as before. At the end of the experiment, the ovarian tissue was homogenized in 3 mL of 0.4 *N* perchloric acid. The resulting suspension was centrifuged at 15,000*g* for 10 min; the supernate contained the [$^3$H]-labeled catecholamines remaining in the tissue. Portions (0.6 mL) from each 1-min fraction and from the tissue homogenates were counted for radioactivity (52% efficiency) in a scintillation counter. The overflow of radioactivity was calculated as a percentage of the total fractional release, i.e., as a percentage of the radioactivity present in the tissue *(16)*.

### *Assay of β-Adrenergic Receptors in Ovarian or Granulosa Cell Membranes*

Membranes were prepared from ovarian tissue (60 mg) or from granulosa cells by differential centrifugation *(22)*. The assay contained 0.5–4.0 n*M* [$^3$H]dihydroalprenolol (92.0 Ci/mmol; Dupont/NEN) with membranes (70 μg) in a total volume of 0.2 mL. Nonspecific binding was assessed in tubes containing $10^{-4}$ *M* DL-propranolol. Competition experiments were as described above but with 2 n*M* DHA with $10^{-9}$–$10^{-3}$ *M* propranolol, nadolol (a $\beta_1$-antagonist), or zinterol (a $\beta_2$-agonist). Results are expressed as femtomoles of dihydroalprenolol bound/milligram of protein per 30 min. Samples were incubated for 30 min at 37ºC. Binding was terminated by the addition of 10 vol of assay buffer and vacuum filtration through Whatman (Clifton, NJ) GF/C fiberglass filters as described *(22)*. Radioactivity retained on the filters was determined by liquid scintillation counting.

### *Statistical Analyses*

Binding parameters ($K_d$, $B_{max}$) were obtained using the ISIS-59 and SCATFIT-59 computer programs. $IC_{50}$ values were determined by log-logit graphic analysis. Differences between two groups were tested by student's *t*-test.

## Acknowledgments

We thank Dr. C. I. Pogson for his help in the preparation of the manuscript. This work was supported by Proyecto DID Multidisciplinario en Salud 002/2, Universidad de Chile.

## References


1. Burden, H. W. (1985). In: *Catecholamines as hormone regulator.* Ben-Jonathan, N., Bahr, J. M., and Weiner, R. I. (eds.). Serono Symposia Publications. Raven: New York.
2. Owman, C. H., Rosengren, E., and Sjöberg, N.-O. (1967). *Obstet. Gynecol.* **30,** 509–514.
3. Moshin, S. and Pennefather, J. N. (1979). *Clin. Exp. Pharmacol. Physiol.* **6,** 335–354.
4. Ojeda, S. R. and Lara, H. E. (1989). In: *The menstrual cycle and its disorders*. Pirke, K. M., Wuttke, W., and Scheiwerg, U. (eds.). Springer-Verlag: Berlin.
5. Mayerhofer, A., Dissen, G. A., Costa, M. E., and Ojeda, S. R. (1997). *Endocrinology* **138,** 3320–3329.
6. Lara, H. E., Ferruz, J. L., Luza, S., et al. (1993). *Endocrinology* **133,** 2690–2695.
7. Owman, C. H., Kannisto, P., Liedberg, F., Schmidt, G., Sjöberg, N.-O., Stjernquist, M., and Walles, B. (1992). In: *Local regulation of the ovarian function*. Sjöberg, N.-O., Hamberger, L., Janson, P. O., Owman, C. H., and Coelingh Bennink, H. J. T. (eds.). Parthenon Publishing Group: NJ.
8. Owman, C. H., Sjöberg, N.-O., Svenssin, K.-G., and Walles, B. J. (1975). *Reprod. Fertil.* **45,** 553–556.
9. Walles, B., Falck, B., Owman, C. H., and Sjöberg, N.-O. (1977). *Biol. Reprod.* **17,** 423–431.
10. Webley, G. E., Luck, M. R., and Hearn, J. P. (1988). *J. Reprod. Fertil.* **84,** 669–677.
11. Hillensjö, T., Sjögren, A., Strander, B., et al. (1985). *Acta Endocrinol. (Copenh.)* **110,** 401–407.
12. Richardson, M. C. and Masson, G. M. (1980). *J. Endocrinol.* **87,** 247–254.
13. Wakade, A. R. (1979). *Nature* **281,** 374–376.
14. Hughes, J. and Roth, R. H. (1974). *Br. J. Pharmacol.* **51,** 373–381.
15. Lara, H. E. and Belmar, J. (1991). *Biol. Reprod.* **44,** 752–759.
16. Ferruz, J., Barria, A., Galleguillos, X., and Lara, H. E. (1991). *Biol. Reprod.* **45,** 592–597.
17. Jacobowitz, D. and Wallach, E. E. (1967). *Endocrinology* **81,** 1132–1139.
18. Bodis, J., Hartmann, G., Török, A., et al. (1993). *Exp. Clin. Endocrinol.* **101,** 178–182.
19. Mayerhofer, A., Hemmings, H. C. Jr., Snyder, G. L., et al. (1999). *J. Clin. Endocrinol. Metab.* **84,** 257–264.
20. Porcile, A., Gallardo, E., and Venegas, E. (1990). *Fertil. Steril.* **53,** 50–55.
21. Shah, S. D., Clutter, W. E., and Cryer, P. E. (1985). *J. Lab. Clin. Med.* **106,** 624–629.
22. Lara, H. and Bastos-Ramos, W. J. (1988). *Neurosci. Res.* **19,** 239–244.
23. Südhof, T. H. (1995). *Nature* **375,** 645–653.
24. Semenova, II. (1969). *Vest. Akad. Med. Nauk. SSSR* **24,** 58–62.
25. Paredes, A., Galvez, A., Leyton, V., et al. (1998). *Endocrine* **8,** 309–315.
26. Aguado, L. I., Petrovic, S. L., Ojeda, S. R. (1982). *Endocrinology* **110,** 1124–1132.
27. Eyster, K. M. and Stouffer, R. L. (1985). *Endocrinology* **116,** 1552–1558.
28. Mayerhofer, A., Fritz, S., Grunert, R., et al. (2000). *J. Clin. Endocrinol. Metab.* **85,** 4750–4757.
29. Barria, A., Leyton, V., Ojeda, S. R., and Lara, H. E. (1993). *Endocrinology* **133,** 2696–2703.
30. Saller, C. and Zigmond, M. (1978). *Life Sci.* **23,** 1117–1130.