1989

The Magnetic Separation of Rat Anterior Pituitary Cells

Jodi Flaws
Loyola University Chicago

THE MAGNETIC SEPARATION OF RAT ANTERIOR PITUITARY CELLS

by

Jodi Flaws

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

March

1989

ACKNOWLEDGMENTS

I give my warmest thanks to my advisor, Dr. Diane Suter, for her support, encouragement, motivation, guidance, and friendship. I am gratefully indebted to Dr. Suter for passing on her love of research to me. I also give warmest thanks to the other members of my committee, Dr. Howard Laten and Dr. Frederick Wezeman, for all of their time and suggestions which made this thesis project possible. I am thankful for all of the help that I received from Adil Javed, David Brashinger, and Eva Gornekevich. I am also thankful for the support and understanding of Patricia Tokimoto, Ray Federico, Meg Graham, and Malini Chawla. Without them, graduate school would not have been nearly as much fun. I am grateful to Dr. Jan Roser for providing the antibody used in this project. I am especially grateful to Dr. Harold Papkoff for sharing some of his wisdom with me and for teaching me that science is truly wonderful. Finally, I dedicate this thesis to my family and John Siwek for their love and support. Without them, none of this thesis project would have been possible.

VITA

The author, Jodi Anne Flaws, is the daughter of Genevieve Flaws. She was born October 26, 1964 in Chicago, Illinois.

Her elementary education was obtained in the public schools of Chicago, Illinois. Her secondary education was completed in 1982 at Reavis High School, Burbank, Illinois.

In September 1982, Jodi entered St. Xavier College, receiving the degree of Bachelor of Science in biology in January, 1986.

In September 1987, Jodi was granted an assistantship in biology at Loyola University of Chicago, enabling her to complete the Master of Science degree in 1990.

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INTRODUCTION

The anterior pituitary gland is known to play an important role in controlling many endocrine glands such as the adrenals, thyroid, mammary glands, and the gonads. The anterior pituitary gland secretes at least eight different hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), prolactin (PRL), beta-endorphin, alpha-melanocyte stimulating hormone (alpha-MSH), and adrenocorticotrophic hormone (ACTH). LH and FSH regulate many parts of the reproductive system. For example, LH stimulates corpora lutea formation and ovulation in the female and testosterone secretion in the male. FSH is responsible for early development of the ovarian follicle in the female and initial spermatid maturation in the male.

Although most of the pituitary hormones are localized to one cell type in the pituitary, LH and FSH are localized in one of three cell types: cells that contain only LH, only FSH, or both LH and FSH. It is important to separate LH- and FSH-containing cells (gonadotropes) from a mixed population of anterior pituitary cells so that several questions can be addressed. For example, it has

been shown that gonadotropin-releasing hormone (GnRH), which is secreted from the hypothalamus, will bind to receptors on the gonadotropes and cause the release of LH as well as FSH from the gonadotropes. It is not known, however, if GnRH causes the release of FSH from cells that contain only FSH or if GnRH causes FSH release only from cells that contain both LH and FSH. Once a pure cell population is achieved, it will be possible to treat cells that contain only FSH with GnRH in order to determine whether or not GnRH will cause the release of FSH from the cells or if another releasing factor is responsible for FSH release from these cells. This information would allow a better understanding of the feedback loops involved in reproduction and help to explain the nonparallel release of LH and FSH in response to steroid hormones such as estradiol and progesterone during the menstrual or estrous cycle.

A pure cell population of gonadotropes would also help address questions about the types of second messenger systems that control hormone release. It is important to understand which second messenger systems are involved in hormone release so that the mechanism of action of the hypothalamic hormones and the way a cell is differentially regulated can be better understood. It is not yet known if the same second messenger system is responsible for the release of LH or FSH from cells. These questions will

remain unanswered until the second messenger system of pure populations of cellscontaining only LH, only FSH, or both hormones can be studied without interference from other cell types.

There is also evidence that cells that contain FSH may also contain ACTH. With a pure population of cells containing only FSH, it would be possible to measure the cells for ACTH content.

Once a valid separation procedure is established, as this thesis project proposes, it will become possible to address some of the aforementioned questions as well as others. Once some of these questions are answered, a better understanding of the reproductive system will be achieved.

SPECIFIC AIMS

1. To develop a rapid, inexpensive, and reliable method to separate cells that contain and secrete LH from a mixed population of rat anterior pituitary cells.

2. To use this new method to support the hypothesis that LH and FSH are secreted from pituitary cells that contain only LH, only FSH, or both LH and FSH.

LITERATURE REVIEW

The anterior pituitary gland is known to secrete at least eight different hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), prolactin (PRL), beta-endorphin, alpha-melanocyte stimulating hormone (alpha-MSH), and adrenocorticotrophic hormone (ACTH). It is important to study the secretion of these various hormones to understand the function and dysfunction of almost all parts of the endocrine system, since the pituitary hormones regulate endocrine organs in many parts of the body. LH and FSH in particular are responsible for control of reproductive function via the gonads, and thus are referred to as gonadotropins. LH is known to stimulate corpora lutea formation and ovulation in the female and to stimulate testosterone secretion in the male. This testosterone secretion causes the development of male secondary sexual characteristics such as hair growth and distribution, enlargement of the sex organs, and development of horns and antlers in some species.[1] FSH is known to stimulate follicular growth in the ovaries and is necessary to increase spermatogenic activity in the testes.[1,2] As early as 1954, electron microscopic studies

showed that the anterior pituitary cell population contains at least two different types of gonadotropes (gonadotropin-secreting cells). Farquhar and Rinehart were able to show with electron microscopy that one type contained rounded, light vesicular nuclei and ovoid cytoplasmic vesicles, whereas a second type contained dense, elongated or folded nuclei and irregularly shaped vesicles separated by cytoplasmic strands.[3] Through this work, Purves and Griesbach determined that one type of cell was primarily located near the periphery of the pituitary gland and that the other type of cell was located centrally.[3] In order to determine which cell type secreted LH and which cell type secreted FSH, Purves and Griesbach treated adult female rats with testosterone and then observed the pituitary cells from these rats via electron microscopy. The electron micrographs showed that the peripheral cells had increased in size and that the central cells had been reduced in size. Purves and Griesbach concluded that the peripheral cells were FSH gonadotropes since testosterone had been previously shown to cause an increase in FSH secretion. They also concluded that the central cells were LH gonadotropes since testosterone had been previously shown to cause a decrease in LH secretion.[3,4,5]

Although these early studies do suggest that LH gonadotropes are centrally located and that FSH gonado-

tropes are peripherally located, due to the lack of immunocytochemical techniques, scientists were unable to show definitely that the hormone contained in or secreted from the centrally or peripherally located cells was LH or FSH. In addition, the aforementioned technique was unable to detect pituitary cells that contained both LH and FSH.

Several immunocytochemical studies have shown that some pituitary gonadotropes exhibit morphologies in which a single cell can contain both FSH and LH.[5,6,7] One of the earliest immunocytochemical studies performed by Nakane involved the use of double-staining anterior pituitary cell populations for both LH and FSH. From this study, Nakane concluded that a high percentage of gonadotropes present in the anterior pituitary contained both FSH and LH.[6] The results of these immunocytochemical studies caused scientists to dispute the "one-cell-one-hormone" hypothesis, which states that each individual cell produces and secretes only one hormone.

These studies raised several important questions regarding the way that LH and FSH from the same cell are differentially regulated in response to only one releasing hormone (gonadotropin-releasing hormone). For example, it is possible that in addition to gonadotropin releasing hormone (GnRH), other releasing hormones exist which may serve to cause the release of hormone from only FSH-containing cells, LH-containing cells, or cells that

contain both LH and FSH.

To study the biochemical mechanisms controlling secretion in specific cell types, a pure cell population is ultimately required. Thus, it is necessary to develope an effective method of separating mixed populations of anterior pituitary cells. A technique known as velocity sedimentation at unit gravity has been used to obtain enriched pituitary gonadotrope populations. This technique employs the theory that the majority of the hormone contained within a pituitary cell is stored in secretory granules of varying size and density. Thus, cells that are more dense due to containment of larger and more dense secretory granules will sediment at a higher density in a gradient made of 0.3-2.4% bovine serum albumin (BSA) maintained at unit gravity than cells that are less dense. The more dense cells can then easily be separated from cells containing smaller and less dense secretory granules. [8,9,10,11] Although this procedure can provide a 6 to 8-fold enrichment of gonadotropes, several other limiting factors exist. Gradient instability can occur because of convection currents generated by improper lighting or by room vibrations, and cell-cell interactions have to be avoided by using only a small number of cells.[8,9] It is also critical to use only a small number of cells during this procedure since when too many cells are layered over a BSA gradient, ideal sedimentation no

longer occurs. Once ideal sedimentation no longer occurs, predictable cell separation is no longer possible.[9] In addition, it has been shown that this method does not allow separation of adult rat pituitary cells into pure gonadotrope populations due to their tendency to spread along a gradient.[12]

In an attempt to eliminate some of the problems encountered with velocity sedimentation at unit gravity, a process known as centrifugal elutriation was developed. During this procedure, dispersed pituitary cells are centrifuged in an elutriator chamber, causing the cells to settle toward the bottom of the chamber due to centrifugal force, while buffer is pumped upward through the chamber, causing the cells also to move upward. Thus, the cells are forced to form a layered gradient. The cells in the chamber are then collected in fractions by increasing the flow rate of buffer through the bottom of the elutriator chamber. This procedure will allow 300 million cells to be collected and separated on the basis of size with a 78% or better recovery and thus provide an adequate number of enriched cells to be obtained for further studies.[12] Centrifugal elutriation is also rapid compared to velocity sedimentation at unit gravity, requiring only 80 minutes to separate and collect cells, compared with 4 hours or more by velocity sedimentation at unit gravity.[8,11] This procedure, however, still lacks the resolution necessary

for studies of biochemical mechanisms for gonadotropin secretion since it does not produce pure cell populations.[8]

The third technique developed for separating mixed cell populations is known as fluorescence-activated cell-sorting. In this procedure, fluorescent dyes are covalently bound to antibodies that are directed against a cell-surface antigen such as a receptor or other protein. A fluorescence-activated cell-sorter is then used to separate fluorescently labeled cells from unlabeled cells.[13,14,15,16] Thorner et al. have shown with this method that pituitary cells that are bound to anti-LH fluorescent microspheres can be separated from a mixed pituitary population to obtain an enriched fraction of gonadotropes.[13] This population was enriched from a normal control range of 7.4 $\pm$ 1.4% to 52.3 $\pm$ 11% of sorted cells.[13] This procedure tends to be relatively inefficient because only 29.5 $\pm$ 3.2% of the total rat anterior pituitary cell population can be recovered from the cell sorter.[13] This procedure is also quite expensive since it requires the availability of a fluorescence-activated cell-sorter.

Another technique that has proven useful in studying secretion from specific cell types is the reverse hemolytic plaque assay.[17,18,19] This technique employs the assumption that a particular hormone will be radially

dipensed around the cell that secreted it and that this region can be visualized. During this procedure, monodispersed pituitary cells and a suspension of protein-A coated ovine erythrocytes are incubated in a chamber constructed of a poly-L-lysine-coated glass microscope slide. This incubation allows the pituitary cells to attach to the chamber. After attachment, the incubation chamber is then filled with an appropriate hormone-specific antiserum. Once the antiserum has attached to the specific hormone, the protein-A coated erythrocytes that were in suspension with the monodispersed pituitary cells tend to attach to the complex since protein-A binds to gammaglobulins present in the antiserum. Complement is then added to the chamber, which causes lysis of the hormone bound protein-A coated ovine erythrocytes. This lysis then causes the formation of areas of hemolysis (reverse plaques). Plaques are then visualized and measured with a calibrated ocular micrometer. In addition, the calibrated ocular micrometer can be used to quantitate the amount of a particular hormone present. To date, this technique has been used to observe secretion of LH, GH, and Prl, but only one abstract has been published on use of this technique to observe FSH secretion.[17,18,19] Although this method is a simple, rapid, and sensitive method for detecting hormone secretion by a single cell, it may not prove useful for the study of FSH since it is

difficult to obtain anti-FSH antibodies suitable for use with immunological techniques.[20]

Another technique that has been used to sort living pituitary cells is known as forward angle and perpendicular light scatter.[21,22] During this procedure, pituitary cells are separated using a cell sorter in which an argon laser is used to generate a light scatter perpendicular to a pituitary cell population. In addition, a forward angle light scatter is also generated from a pituitary cell population. It is generally agreed that the forward angle light scatter will diffract light dependant on the size of the cell. It is also generally agreed that perpendicular light scatter will cause a reflection and refraction of light that is dependant on internal structures of the cell such as the ratio of nuclear-cell diameter, cytoplasmic secretory granule content, nuclear shape, and distribution of cytoplasmic organelles. Therefore, if one assumes that the size and internal structure of the cells to be separated differ from the rest of the cell population, separation can be achieved. There are basically two major drawbacks to this type of a separation procedure: 1) the assumption that size and internal structure differs among pituitary cells and 2) the cost of a cell-sorter.

Recently, separation techniques have been developed that use magnetic principles to separate cells into pure

fractions. Early magnetic separation techniques could only be performed on red blood cells, which are intrinsically magnetic, or on phagocytic cells, which could ingest magnetic beads. Magnetic particles now can be attached via antibodies to cell surfaces to render them magnetic, since all cell types have specific markers or proteins on their surfaces.[23,24,25,26] Cells attached to magnetic particles can then be separated from cells not attached to magnetic particles by simply applying a magnetic field. This procedure has been used successfully to separate CD8+T cells from peripheral blood suspensions and tumor cells from bone marrow.[18,20,27,28] To date, the use of magnetic separation techniques to separate mixed populations of anterior pituitary cells has been unreported. When this procedure is applied to rat anterior pituitary cells, as this thesis project proposes, magnetic beads coated with an anti-LH or anti-FSH antibody will be used to separate LH-secreting or FSH-secreting cells from the rest of the cell population. Therefore, once the separation of gonadotropes from a mixed population of rat anterior pituitary cells has been achieved, the hypothesis for this thesis project will be that LH and FSH are secreted from pituitary cells thatcontain only LH, only FSH, or both LH and FSH.

MATERIALS AND METHODS

Activation of magnetic beads

In all experimental procedures, the magnetic beads used were made of polystyrene with a magnetite core and a diameter of 4.5 um (Dynabeads^{R}). I activated the magnetic beads according to a procedure outlined by the manufacturer (Dynal Inc.) to prepare the surface of the beads for conjugation to an antibody. I first subjected the magnetic beads to a series of washes with acetone, in order to remove water from the magnetic beads. In order to transfer the magnetic beads to acetone, I first suspended the beads in a 7:3 water:acetone mixture (1.5 ml mixture/4.8 x 10^{8} magnetic beads). I then collected the magnetic beads by centrifugation for 10 min at 23,000 g or with a magnetic particle collector for 5 min. After collection of the magnetic beads, I resuspended the magnetic beads in a 6:4 water:acetone mixture for 5 minutes. I then collected the magnetic beads as above and resuspended them in a 2:8 water:acetone mixture for 5 minutes. After collection of the magnetic beads again, I resuspended the magnetic beads in 1.5 ml acetone for 5 minutes. After this resuspension, I recollected the magnetic beads. I performed the resuspension in 1.5 ml

fresh acetone 3 times. Once the washes were completed, I resuspended the magnetic beads in 1.5 ml acetone and added 0.75 mmol pyridine and 1.5 mmol p-toluenesulphonyl chloride/30 mg undiluted magnetic beads. I then incubated the mixture for 20 h with end-over-end rotation at room temperature. After incubation, I collected the magnetic beads by centrifugation or with a magnetic particle collector as described above and washed the magnetic beads 3 times in 1.5 ml acetone. After each wash, I collected the magnetic beads as previously mentioned. I then transferred the beads back to water through a series of washes in which I sequentially resuspended the magnetic beads in 1.5 ml of 2:8, 6:4, and 7:3 water:acetone mixtures. I once again collected the magnetic beads by the aforementioned methods. Finally, I stored the now-activated magnetic beads in 1 mM HCl until needed for further use. The manufacturer of these magnetic beads has determined that activated magnetic beads can be stored for 12 months in 1 mM HCl at 4^{o}C.

Conjugation of magnetic beads

I conjugated the activated magnetic beads to a suitable antibody according to a procedure recommended by the manufacturer. The anti-LH antibody that I used was anti-bLH-518-b7, a monoclonal anti-LH antibody kindly provided by Dr. Jan Roser of the University of California

at Davis. In addition, I also conjugated normal mouse serum (NMS) to magnetic beads as a negative control. NMS was used as a negative control since anti-bLH-518-b7 was raised in a mouse and since NMS does not bind specifically to LH or FSH. According to the recommended procedure, I first washed the activated magnetic beads 3 times in 1 ml sterile distilled water. I then resuspended the magnetic beads in 1 ml sterile distilled water. I diluted NMS or anti-LH antibody in 0.2 M borate buffer, pH 9.5, to obtain a final concentration of 150 ug protein/ml. I added diluted NMS or anti-LH antibody to the washed magnetic beads to achieve an antibody:magnetic bead ratio of 75 ug:15 mg and incubated this mixture for 24 h by slow end-over-end rotation at room temperature. Once the incubation period was over, I collected the magnetic beads with a magnetic particle collector for 5 min or by centrifugation at 23,000 g for 10 min. I then washed the magnetic beads with 1.5 ml 0.1 M phosphate-buffered saline (PBS) pH 7.4 for 10 min.; 1.5 ml 1 M ethanolamine-HCl, pH 9.5, with 0.1% (vol/vol) Tween 20 for 2 h; 1.5 ml 0.05 M Tris-Cl with 0.1 M NaCl, 0.1% (wt/vol) bovine serum albumin (BSA), 0.01% merthiolate, and 0.1% Tween 20, pH 7.5, for 12 h; and 1.5 ml 0.05 M Tris-Cl with 0.1 M NaCl, 0.1% BSA, and 0.01% merthiolate, pH 7.5, for 2 h. I then collected the NMS-coated or antibody-coated magnetic beads by centrifugation or with a magnetic particle collector

and stored them in 0.05 M Tris-Cl, pH 7.4, with 0.1 M NaCl, 0.1% BSA, and 0.01% merthiolate until needed for further use.

Specificity check for the binding of anti-LH antibody to the magnetic beads

I ran two standard tests to ensure that anti-LH antibody was bound to the magnetic beads and that the binding of the antibody-coated beads to antigen was specific. I ran test one to show specificity through a dose-response curve and test two to show specificity through a dose-inhibition curve. A dose-response curve exhibits specificity by showing that binding of radiolabeled hormone to a specific antibody increases as the concentration of the antibody increases. A dose inhibition curve exhibits specificity by showing that radiolabeled hormone and nonradiolabeled hormone will compete for binding to antibody against the hormone. I ran these tests according to procedures A and B below:

A. **Specificity check 1**

During this procedure I incubated 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 ul magnetic beads conjugated to anti-bLH-518-b7 (518b7db) with ^{125}I-LH (50,000 cpm/50 ul) and PBS, pH 7.0, with 0.1% (wt/vol) gelatin (PBS-gel) in a

total volume of 150 ul. In addition, I also incubated magnetic beads conjugated to NMS (NMSdb) with PBS-gel and ^{125}I-LH as a control to determine the amount of non-specific binding of radioactive hormone to the magnetic beads. I performed the incubation for 24 h at room temperature in 1% gelatin-coated disposable glass culture tubes. The gelatin helped to prevent proteins from binding to the glass. After the incubation period, I added 2 ml cold PBS to each tube and centrifuged the tubes at 1700 g for 2 h. I then decanted the supernatants and washed the tubes with an additional 2 ml cold PBS. I then used an automatic gamma counter to determine the amount of bound radiolabeled hormone on the dynabeads in the tube. I replicated this experiment three times.

B. Specificity check 2

During this procedure, I incubated a constant concentration of 518b7db (0.1 ul) and 50 ul ^{125}I-LH (50,000cpm/50 ul) with various concentrations of non-radiolabeled LH (0.02, 0.06, 0.2, 0.63, 2.01, 6.34, 20.03, 63.3, and 200.0 ng/200 ul) and PBS-gel or non-radiolabeled FSH (0.30, 0.59, 1.19, 2.38, 4.75, 9.5, 19.0, and 38.0 ng/200 ul) and PBS-gel in a total incubation volume of 475 ul. In addition, I incubated tubes containing NMSdb (0.1 ul) with 50 ul radiolabeled hormone and the various concentrations of non-radiolabeled hormone as described

above. The incubation took place in 1% gelatin-coated glass culture tubes at room temperature for a period of 24 h. After the incubation period, I washed the tubes and determined the amount of bound radiolabeled hormone as described in specificity check 1. I replicated this experiment three times.

Time Course Binding Study

I ran a time course binding check to determine the amount of time that antibody remains conjugated to the magnetic beads. During this procedure, I incubated 0.1 and 0.2 ul 518b7db or NMSdb with ^{125}I-LH (50,000 cpm/50 ul) and PBS-gel in a total volume of 150 ul. In addition, I incubated 200 ul 1:400 normal rabbit serum (NRS) in ethylene diamine tetraacetic acid-PBS (EDTA-PBS) or 1:45,000 anti-rLH-S9 in NRS with 500 ul PBS-gel and ^{125}I-LH (100,000 cpm/100 ul). These tubes served as a control to determine the amount of binding lost over time due to decay of the radioactive hormone. Therefore, if the ratio of specific binding of ^{125}I-LH to 518b7db : specific binding of ^{125}I-LH to anti-rLH-S9 remained constant over time, I would show that binding was not lost over time due to the antibody dissociating from the magnetic beads. I incubated the tubes for 24 h at room temperature. After the incubation period, I added 50 ul protein-A (1 mg IgG binding capacity/ml) to the control tubes, incubated at

4°C for 15 min, and then washed and centrifuged all the tubes as previously described. I then determined the amount of bound radiolabeled hormone as described in specificity check 1. I performed this procedure once a week for 5 weeks.

Radioimmunoassay (RIA)

I used assays for LH, FSH, TSH, and Prl according to standard laboratory procedures with reagents obtained from the National Pituitary Agency.[29,30,31,32,33,34] I radioiodinated all hormones by the chloramine T method. The intra-assay coeffients of variation (CVs), inter-assay CVs, and upper and lower limits of detectability are described in Table 1.

Table 1 : RIA CV's and limits of detectability

I determined intra-assay and inter-assay coefficients of variation for LH, FSH, TSH, and Prl radioimmunoassays (RIA) that I used in Experiments 1 and 2. I also determined the upper and lower levels of detectability for the aforementioned assays.

Table 1

Hormone	Intra-assay CV	Inter-assay CV	Lower level ng/tube	Upper level ng/tube
LH	5.9%	6.5%	0.125	4.0
FSH	4.2%	7.2%	1.19	4.75
TSH	10.1%	19.1%	0.122	3.4
Prl	10.1%	11.2%	0.176	9.24

Experiment 1

Once the aforementioned procedures were followed, I used antibody-coupled beads in the following methods to separate freshly dissociated rat anterior pituitary cells. I replicated Experiment 1 and Experiment 2 three times.

A. Animals

I used female rats at least six weeks of age that were bred in Loyola University of Chicago's animal care facility for all the following experimental procedures. This animal care facility is APHIS-accredited and staffed by a full-time animal care technician. The temperature in the facility was maintained at 22-25^{o}C and the light cycle was maintained with 12 h dark:12 h light. The animals were allowed food and water ad libitum.

B. Cell culture

I enzymatically dissociated 30 rat pituitaries according to standard laboratory techniques.[35,36] Once the cells were dissociated, I added culture medium [Dulbeccos Modified Eagles Medium (DMEM), 10% (vol/vol) fetal bovine serum, 100,000 U/ml mycostatin, and 1%

(vol/vol) penicillin-streptomycin] to a final volume of 15 ml. I then removed 2.6 x 10^6 cells from the final dissociated population, which I plated at 200,000 cells/well (1.5 ml/well) in 16 mm culture dishes. These cells served as control cells. I then had approximately 52.4 x 10^6 cells left. I placed 1 x 10^7 cells into each of three separate sterile siliconized culture tubes with rubber stoppers. To transfer the cells to the tubes I gently pipetted the correct volume of cells into three separate tubes. One of these tubes received 518b7db and was separated as described below. One tube did not receive 518b7db and served as a control for the incubation periods and treatments described below (incubated-only cells). The third tube received 518b7db, but was not separated as described below (nonseparated cells). This tube served as a control for the presence of magnetic beads during the incubation periods and treatments described below.

C. Addition of magnetic beads and GnRH

I pipetted 1.0 x 10^8 518b7db (0.250 ml) into the tube that contained the cells to be used for the separation procedure and into the tube that contained cells that were to serve as nonseparated control. This number of magnetic beads was ten times the number of cells, so that a negative selection could be achieved. This excess should remove most of the cells secreting LH from the

mixed cell population, but may also remove some non-LH-secreting cells. I then added GnRH to the tubes in a final concentration of 10^{-9} M to cause maximal secretion of gonadotropins from the cells. Once the cells, antibody-coated beads, and GnRH were added to the tubes, I incubated the tubes with an end-over-end rotator at room temperature for 4 h. During this incubation, I aerated the cells with a 95% O_2/5% CO_2 mixture for 30 s every hour.

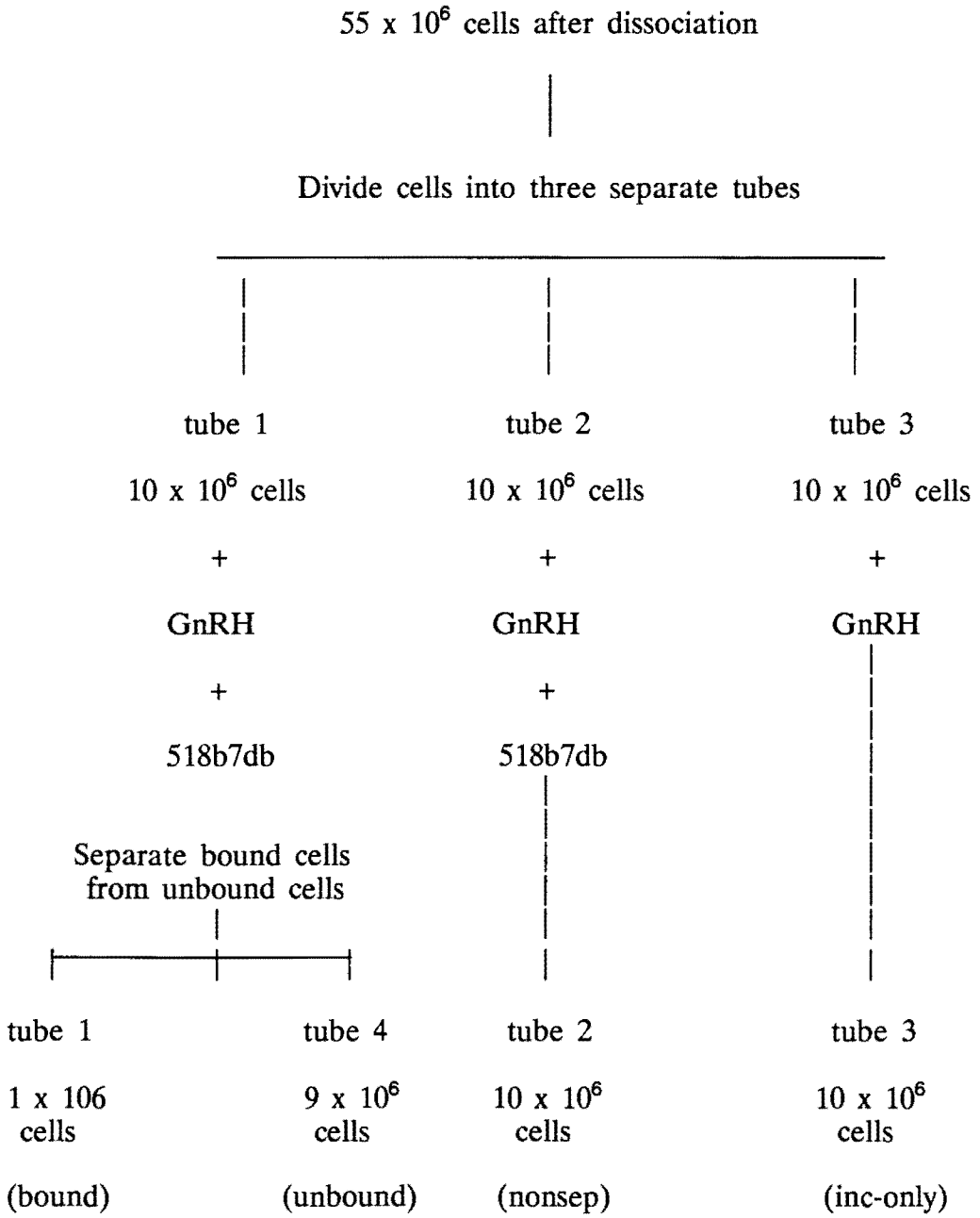
D. Separation of bound cells from unbound cells

After incubation for 4 h, I placed one of the tubes containing 518b7db and GnRH in a magnetic particle concentrator for 5 min to separate bound cells from unbound cells. I then removed the unbound population with a pasteur pipet and placed the cells into a sterile siliconized tube. I then resuspended the bound population in 3 ml culture medium. Thus I was left with 4 tubes containing: bound cells, unbound cells, incubated-only cells, and nonseparated cells. Since the bound cells from each tube would be approximately 10% of the population[4], Table 2 gives a good approximation of the number of cells I expected to have in each tube:

Table 2 : Separation procedure using 518b7db

I separated LH-secreting cells from a mixed anterior pituitary population with the use of 518b7db. I divided a mixed population of cells into 3 separate tubes. I added gonadotropin releasing hormone (GnRH) to each of the tubes and added 518b7db to 2 of the tubes. I then put tube 1 into a magnetic particle concentrator to separate the cells bound to 518b7db from cells that were not bound to 518b7db.

Table 2: Separation procedure using 518b7db



E. Treatment of cells with HEPES buffer

After the above procedure was followed, I treated half of each population with HEPES buffer (25 mM HEPES, 1 mg/ml BSA, 1% penicillin-streptomycin, pH 7.4) with or without trypsin (1 mg/ml). I performed the trypsin treatment in order to dissociate the magnetic beads from the bound cells after separation was completed. In order to add the HEPES buffer with or without trypsin, I centrifuged the tubes in a clinical IEC centrifuge at setting 6 for 5 min. I then aspirated the supernatant with a pasteur pipet. Once the supernatant was aspirated, I then added HEPES buffer with or without trypsin (1 ml solution/2 x 10^6 cells). I then incubated each tube of cells for 40 min at 37^{o}C. After the incubation period, I placed the bound populations of cells that were treated with HEPES buffer with or without trypsin in a magnetic particle concentrator. This procedure separated the bound cells from the magnetic beads. I then transferred the cells to another tube with a pasteur pipet. After this separation, I stopped the trypsin reaction in every tube by adding 8 ml culture medium. I then washed the cells three times with culture medium. To wash the cells, I centrifuged the cells in a clinical IEC centrifuge at setting 6 for 5 min, aspirated the supernatant with a pasteur pipet, added 5 ml culture medium, and gently

resuspended the cells with a pasteur pipet.

F. Plating of cells

I plated the cells from the aforementioned tubes at 200,000 cells/well (1.5 ml/well).

G. Incubation of cells

I then incubated the cells for 48 h at $37^{0}C$ in a water-saturated atmosphere of 95% air-5% CO_2. After 48 h, I removed the medium and washed the cells once with fresh DMEM to remove any free antibody or magnetic beads. I then replenished the wells with fresh culture medium. I incubated the plates again for 24 h.

H. Assays

After incubation for 24 h, I collected the cells and the media and assayed for LH and FSH by RIA. To collect the media, I removed the media from the plates with a pasteur pipet. To collect the cells, I first added lysis buffer to the plates (0.05 M Na_2CO_3, 2 mM EDTA, 45,000 U/g bacitracin, pH 8.5).[37] I then froze and thawed the cells two times. This procedure caused the cells to break apart and release their contents into the buffer. I stored the samples at $-20^{o}C$ until RIA.

Experiment 2

After I determined that the magnetic beads used in the aforementioned experiment could be removed by treating the cells with HEPES buffer without trypsin, I performed the following separation experiment so that I could assay the bound and unbound fractions of cells for LH, FSH, TSH, and PRL. It was necessary to assay both the bound and unbound fractions to show that the separation procedure works. In addition, I used NMSdb as a control for nonspecific binding in the separation procedure. It was necessary to use NMSdb as a control because Experiment 1 did not allow me to show that the separation procedure specifically removed LH-secreting cells due to specific binding of 518b7db to LH-secreting cells.

A. Cell culture

I enzymatically dissociated 30 rat pituitaries according to standard laboratory techniques.[35,36] Once the cells were dissociated, I removed 2 x 10^6 cells, which I plated at 200,000 cells/well. These cells served as control cells. I had approximately 53.5 x 10^6 cells left, which I placed into two separate sterile siliconized culture tubes with rubber stoppers.

B. Addition of magnetic beads and GnRH

I added 1.925 x 10^8 518b7db (0.481 ml) to one tube

and 1.925×10^8 NMSdb (0.481 ml) to the other tube. I added GnRH to both tubes to a final concentration of 10^{-9} M. I then incubated and aerated the tubes as previously described.

C. Separation of bound cells from unbound cells

After the incubation period, I placed each of the two tubes in a magnetic particle concentrator for 5 min. I removed the unbound populations with a pasteur pipet and placed them into two separate sterile siliconized tubes. I resuspended the bound populations in 3 ml culture medium. Thus, I was left with four tubes containing: 518b7db-bound cells, 518b7db-treated cells that remained unbound, NMSdb-bound cells, and NMSdb-treated cells that remained unbound.

D. Treatment with HEPES buffer

After the above procedure was followed, I treated all four tubes with HEPES buffer for 40 min as previously described. After the incubation period, I removed the magnetic beads as previously described. I also washed and plated the cells as previously described.

E. Incubation of cells

I then incubated the cells for 48 h at 37^0C in a water-saturated atmosphere of 95% air-5%CO_2. After 48 h, I washed the cells twice with DMEM. I then treated some cells from each magnetic bead treatment group with different releasing factors. In order to add the different releasing factors to the cells, I replenished the cells with fresh culture medium containing the following releasing factors: gonadotropin-releasing hormone (10^{-9} M), corticotropin-releasing hormone (10^{-8} M), growth hormone-releasing hormone (10^{-8} M), or thyrotropin-releasing hormone (10^{-7} M). I also replenished some cells from each group with fresh challenge medium without releasing factors as a control. The concentrations of releasing hormones that I used were consistent with physiological concentrations found in the literature[38,39,40]. I then incubated the cells for 24 h.

F. Assays

After incubation for 24 h, I collected the cells and media as previously described. I then assayed the samples for LH, FSH, TSH, and Prl by RIA.

Statistics

A. Specificity checks

I calculated the mean $\pm$ SEM for three experiments in

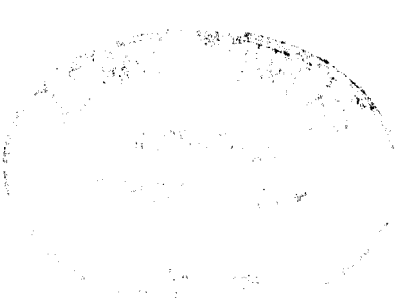
specificity checks 1 and 2.

B. Experiment 1

I analyzed the effect of treatment with HEPES buffer with or without trypsin on the concentration of LH and FSH in media, on LH and FSH cell content, and on total LH and FSH by analysis of variance with trypsin treatment, replicate, and dynabead treatment as the main effects. I used Tukey's test to determine differences between control, bound, unbound, incubated-only, and nonseparated cells.

C. Experiment 2

I analyzed the effect of treatment with magnetic beads and releasing factors on the concentration of LH, FSH, PRL, and TSH in media and within the cells by analysis of variance with releasing factor treatment, replicate, and magnetic bead treatment as the main effects. When a significant F-value was encountered, I used Tukey's test to determine significant differences between treatment means.



Results

A. Specificity check 1 for 518b7db

Fig. 1 shows that specific binding of ^{125}I-LH to 518b7db increased as the concentration of 518b7db increased. This specific binding increased to 34% of total radioactivity. Fig. 1 also shows that specific binding of ^{125}I-FSH to 518b7db did not increase as the concentration of 518b7db increased. The specific binding of ^{125}I-FSH to each concentration of 518b7db remained near 0%.

B. Specificity check 2 for 518b7

Fig. 2 shows that when 518b7db were incubated with a constant amount of ^{125}I-LH and increasing amounts of nonradiolabeled LH, higher amounts of nonradiolabeled LH decreased specific binding from 42% to 5% of total radioactivity. Fig. 2 also shows that when 518b7db were incubated with a constant amount of ^{125}I-LH and increasing amounts of nonradiolabeled FSH, specific binding of ^{125}I-LH to 518b7db remained above 40% of total radioactivity regardless of the concentration of nonradiolabeled FSH used.

C. Time course binding check

Fig. 3 shows that the ratio of specific binding of ^{125}I-LH to 518b7db : specific binding of ^{125}I-LH to anti-rLH-S9 during week 1 was 2.74. During the time course experiment, the ratio did not change.

D. Experiment 1

1. LH medium

Fig. 4 shows the amounts of LH/200,000 cells secreted into the medium by the control, bound, unbound, incubated-only (inc-only), and nonseparated (nonsep) cells. The bound secreted 1.5-fold more LH than controls, 5-fold more than the unbound population, and 2-fold more than the inc-only or nonsep cell populations ($p<.05$). There were no significant differences between the amounts of LH secreted by control, inc-only, and nonsep cells. The unbound population secreted 60% less LH than the control group ($p<.05$). There was no significant difference between the unbound, the inc-only, and the nonsep cells.

2. Cellular LH

Fig. 5 shows the amounts of LH/200,000 cells contained in the control, bound, unbound, inc-only, and non-sep cells. The bound population of cells contained 1.7-fold more LH than control, 7-fold more than the unbound population, 2.5-fold more than the inc-only cells,

and 2-fold more than the nonsep cell populations ($p<.05$). There were no significant differences between the amounts of LH contained in the control, inc-only cells, and nonsep treatment groups. The unbound population contained 76% less LH than the control group ($p<.05$). There were no significant differences between the amounts of LH contained in the unbound, the inc-only, and the nonsep cells.

3. Total LH

Fig. 6 shows total LH/200,000 cells in the control, bound, unbound, inc-only, and nonsep treatment groups. The bound population of cells contained 1.7-fold more total LH than control, 6-fold more than the unbound population, 2.3-fold more than the inc-only cells, and 2.1-fold more than the nonsep cell population ($p<.05$). There were no significant differences between the amounts of total LH in the control, inc-only, and nonsep cells. The unbound population contained 73% less total LH than the control group ($p<.05$). There were no significant differences between the amounts of total LH in the unbound, inc-only, and nonsep treatment groups.

4. Medium FSH

Fig. 7 shows the amounts of FSH/200,000 cells secreted into the medium by the control, bound, unbound,

inc-only, and nonsep treatment groups. The bound population of cells secreted 2.5-fold more FSH than control, 11.2-fold more than the unbound population, and 3.8-fold more than the inc-only and the nonsep cell populations ($p<.05$). There were no significant differences between the amounts of FSH secreted by the control, unbound, inc-only, and nonsep cells.

5. Cellular FSH

Fig. 8 shows the amounts of FSH/200,000 cells contained in the control, bound, unbound, inc-only, and nonsep treatment groups. There were no significant differences between the amounts of FSH contained in any of these treatment groups.

6. Total FSH

Fig. 9 shows the total amounts of FSH/200,000 cells contained in the control, bound, unbound, inc-only, and nonsep treatment groups. There were no significant differences between the total amounts of FSH contained in cells from any of these treatment groups.

E. Experiment 2

1. Effects of releasing factors

The effects of GnRH, TRH, CRH, and GHRH on the different magnetic bead treatment groups were inconsistent and no statistical analyses could be performed.

2. Medium LH

Fig. 10 shows the amounts of LH/200,000 cells secreted into the medium by control, 518b7db-bound cells (b518b7), 518b7db-treated cells that remained unbound (u518b7), NMSdb-bound cells (bNMS), and NMSdb-treated cells that remained unbound (uNMS). B518b7 cells secreted 1.7-fold more LH than control, 2-fold more than bNMS, 3.8-fold more than uNMS cells, and 15-fold more LH than u518b7 cells ($p<.05$). There was no significant difference between the amounts of LH secreted by control, uNMS, and bNMS cells. There also was no significant difference between the amounts of LH secreted by control, u518b7, and uNMS cells. There was, however, a significant difference between the bNMS and u518b7 cells. BNMS cells secreted 7.5-fold more LH than u518b7 cells ($p<.05$).

3. Cellular LH

Fig. 11 shows the amounts of LH/200,000 cells contained in control, b518b7, u518b7, bNMS, and uNMS cells. B518b7 cells contained 2.5-fold more LH than control, 3.8-fold more than uNMS, and 14.8-fold more than u518b7 cells

($p<.05$). There were no significant differences between the amounts of LH contained in control, uNMS, and u518b7 cells. There were also no significant differences between the amounts of LH contained in b518b7 and bNMS cells. BNMS cells contained 10.4-fold more LH than u518b7 and 2.7-fold more than uNMS cells ($p<.05$), but there was no significant difference between bNMS and control cells.

4. Total LH

Fig. 12 shows the amounts of total LH/200,000 cells in control, b518b7, u518b7, bNMS, and uNMS cells. B518b7 cells contained 2.6-fold more total LH than control, 1.65-fold more than bNMS, 3.8-fold more than uNMS, and 14.8-fold more than u518b7 cells ($p<.05$). There were no significant differences between the amounts of total LH in uNMS and control cells. BNMS cells contained 2.3-fold more total LH than uNMS, 9-fold more than u518b7, and 1.6-fold more than control cells ($p<.05$). U518b7 cells contained 74.2% less total LH than uNMS cells and 82.7% less than control cells ($p<.05$).

5. Medium FSH

Fig. 13 shows the amounts of FSH/200,000 cells secreted into the medium by control, b518b7, u518b7, bNMS, and uNMS cells. B518b7 cells secreted 8.6-fold more FSH than u518b7, 2.2-fold more than bNMS, 4.2-fold more than

uNMS, and 2.6-fold more than control cells ($p<.05$). Cells separated using NMSdb (uNMS and bNMS) secreted the same amount of FSH as control cells. FSH secreted by u518b7 cells was not significantly different from control, but was only 12 % of the amount of FSH secreted by bNMS cells ($p<.05$).

6. Cellular FSH

Fig. 14 shows the amounts of FSH/200,000 cells contained in control, b518b7, u518b7, bNMS, and uNMS cells. B518b7 cells contained 3.8-fold more FSH than u518b7, 2-fold more than bNMS, 2.8-fold more than uNMS, and 3-fold more than control cells ($p<.05$). There were no significant differences between the amounts of FSH contained in u518b7, bNMS, uNMS, and control cells.

7. Total FSH

Fig. 15 shows the total amounts of FSH/200,000 cells contained in control, b518b7, u518b7, bNMS, and uNMS cells. B518b7 cells contained 5.4-fold more total FSH than u518b7, 2.2-fold more than bNMS, 3.5-fold more than uNMS, and 2.7-fold more than control cells ($p<.05$). Control cells contained 9 ng FSH/200,000 cells, with bNMS containing 20% more and uNMS containing 30% less ($p<.05$). UNMS cells, however, contained 50% less total FSH than control cells and 1.5-fold more than u518b7 cells ($p<.05$).

8. Medium PRL, cellular PRL, and total PRL

Figs. 16 and 17 show the amounts of PRL/200,000 cells secreted by and contained in control, b518b7, u518b7, bNMS, and uNMS cells. Fig. 18 shows the total amount of PRL/200,000 cells contained in each magnetic bead treatment group. There were no significant differences between the amounts of PRL in any treatment group.

9. Medium TSH, cellular TSH, and total TSH

Figs. 19 and 20 show the amounts of TSH/200,000 cells secreted by and contained in control, b518b7, u518b7, bNMS, and uNMS cells. Fig. 20 shows the total amount of TSH/200,000 cells contained in each magnetic bead treatment group. There were no significant differences between the amounts of TSH in any treatment groups, except control cells contained 1.2-fold more total TSH than uNMS cells ($p<.05$).

Figure 1 : Specificity check 1

During Specificity check 1, I incubated 0.05., 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 ul 518b7db with ^{125}I-LH or ^{125}I-FSH, and PBS-gel for 24 h at room temperature. Specific binding of ^{125}I-LH to 518b7db increased as the concentration of 518b7db increased, while the specific binding of ^{125}I-FSH to 518b7 remained near 0%. All points represent the mean $\pm$ SEM of three experiments.

Figure 1

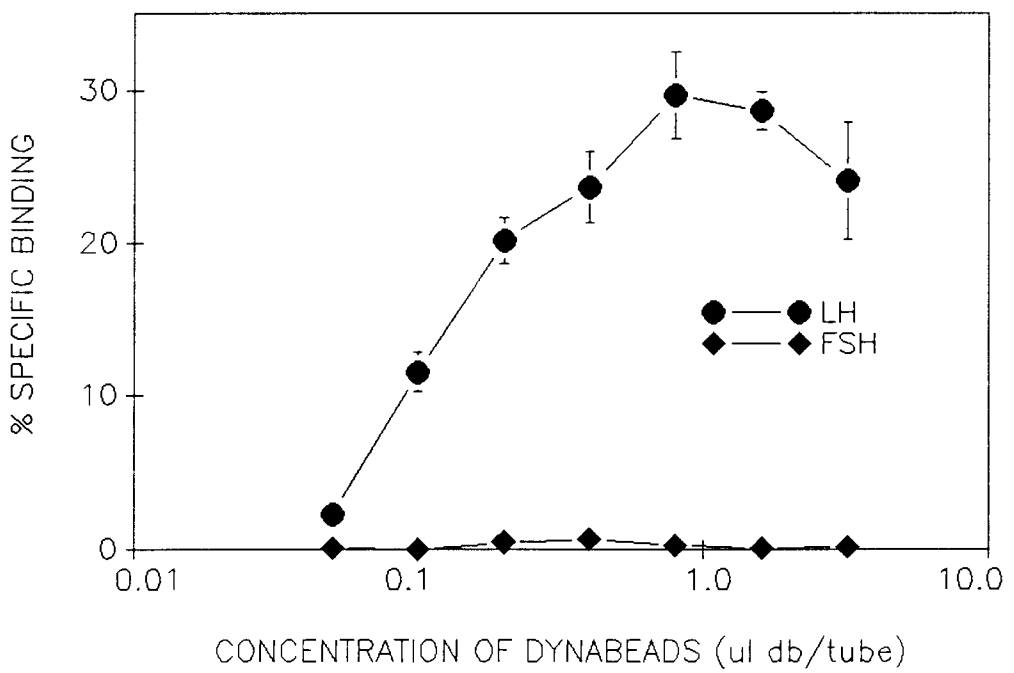

% SPECIFIC BINDING
30
20
10
0
0.01
0.1
1.0
10.0
LH
FSH
CONCENTRATION OF DYNABEADS (ul db/tube)

Figure 2 : Specificity check 2

During Specificity check 2, I incubated 0.1 ul 518b7db with 50 ul ^{125}I-LH and various concentrations of nonradiolabeled LH or FSH for 24 h at room temperature. Nonradiolabeled LH inhibited ^{125}I-LH from binding to 518b7db whereas nonradiolabeled FSH did not. Points represent the mean $\pm$ SEM of three experiments. In some cases, the error bars are smaller than the symbols.

Figure 2

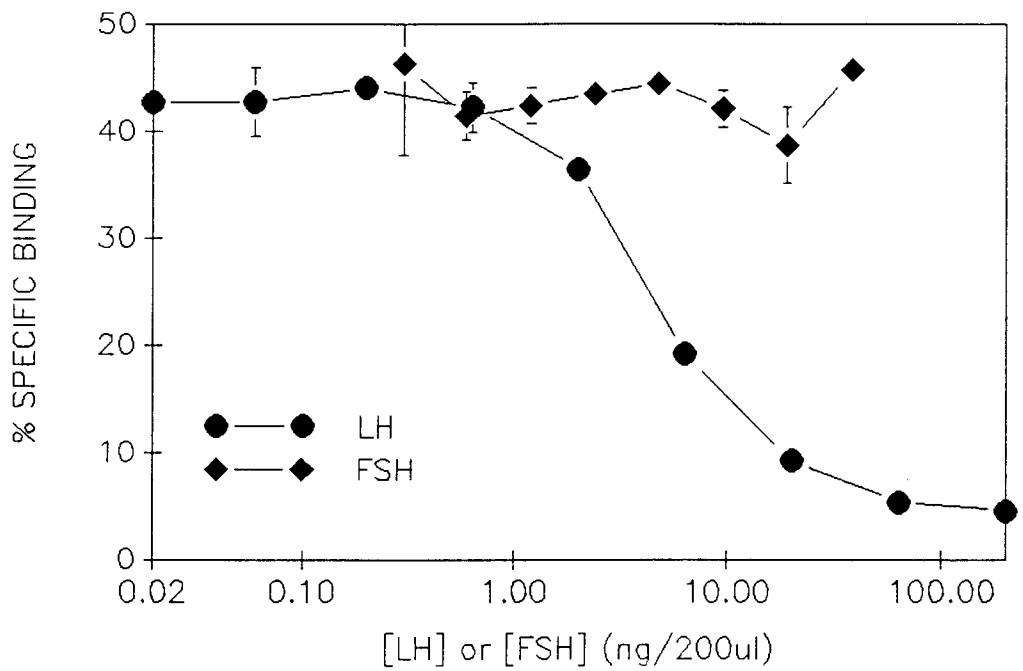

50
40
30
20
10
0
% SPECIFIC BINDING
LH
FSH
0.02
0.10
1.00
10.00
100.00
[LH] or [FSH] (ng/200ul)

Figure 3 : Time-course binding study

During the time-course binding study, I incubated 0.01 ul 518b7db or NMSdb with 50 ul ^{125}I-LH for 24 h. In addition, I also incubated 200 ul NRS or 200 ul anti-rLH-S9 with 100 ul ^{125}I-LH as a control for radioactive decay. I calculated the ratio of specific binding of ^{125}I-LH to 518b7db:specific binding of ^{125}I-LH to anti-rLH-S9 to determine if binding of ^{125}I-LH to 518b7db was decreased over time. During the five week period that the binding checks were performed, there was no loss in binding of ^{125}I-LH to 518b7db.

Figure 3

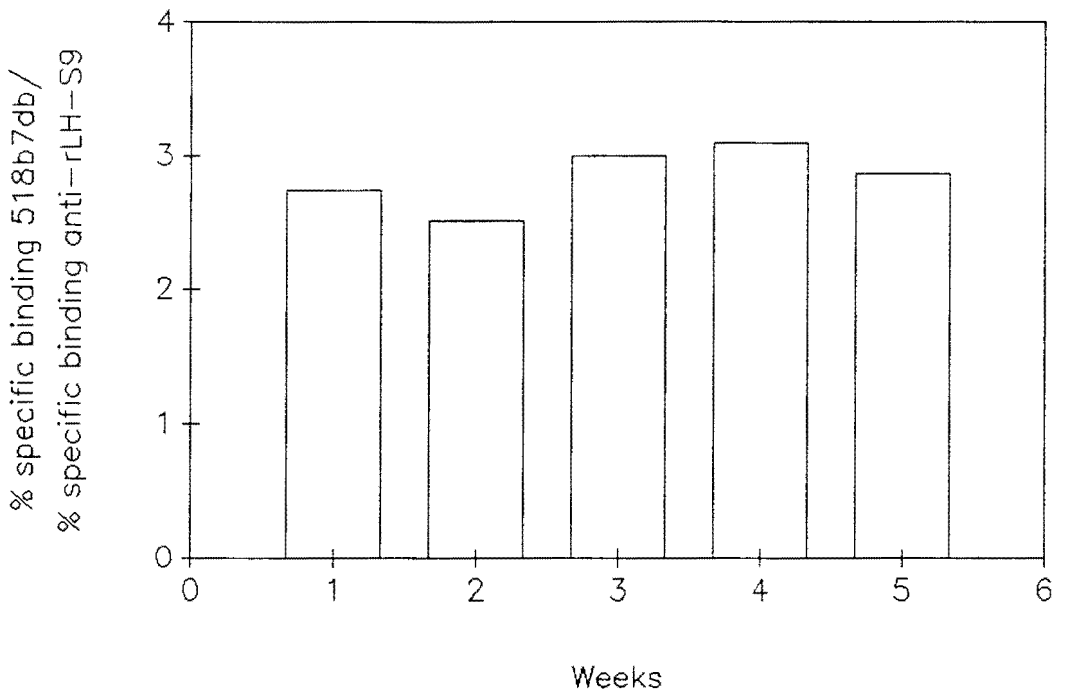

% specific binding 518b7db/
% specific binding anti-rLH-S9
4
3
2
1
0
0
1
2
3
4
5
6
Weeks

Figure 4 : Experiment 1-Medium LH

I measured LH via RIA in medium samples of bound, unbound, incubated-only (inc-only), and nonseparated (nonsep) cells. Treatment groups that were treated with HEPES buffer with or without trypsin were not significantly different from each other and so were combined for presentation here. Bars represent the mean $\pm$ SEM of three experiments with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 4

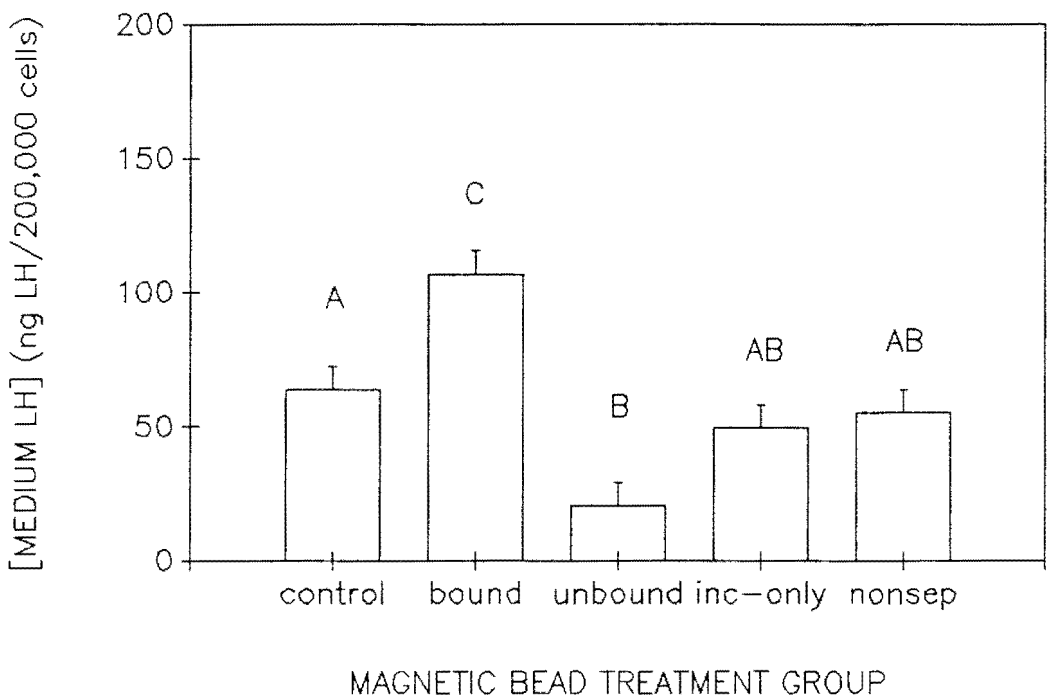

[MEDIUM LH] (ng LH/200,000 cells)
200
150
100
50
0
A
C
B
AB
AB
control
bound
unbound
inc-only
nonsep
MAGNETIC BEAD TREATMENT GROUP

Figure 5 : Experiment 1-Cellular LH

I measured LH via RIA in cell lysates of the bound, unbound, incubated-only (inc-only), and nonseparated (nonsep) cells. Treatment groups that were treated with HEPES buffer with or without trypsin were not significantly different from each other and so were combined for presentation here. Bars represent the mean ± SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 5

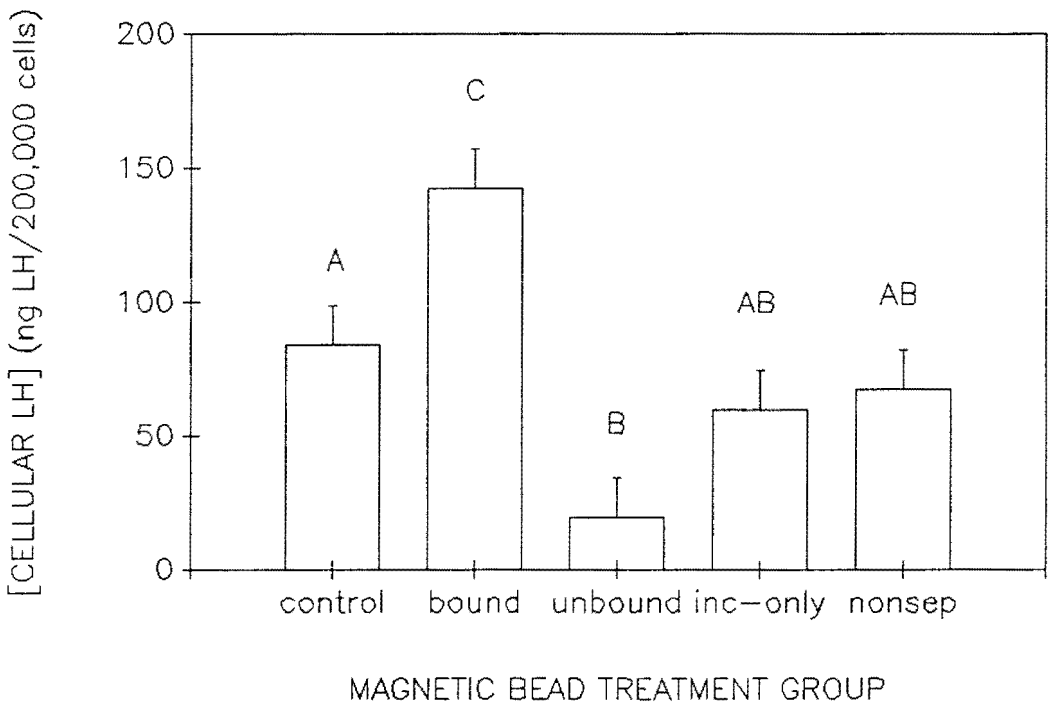

[CELLULAR LH] (ng LH/200,000 cells)
200
150
100
50
0
A
C
B
AB
AB
control
bound
unbound
inc-only
nonsep
MAGNETIC BEAD TREATMENT GROUP

Figure 6 : Experiment 1-Total LH

I calculated total LH by adding medium and cellular LH values obtained in the bound, unbound, incubated-only (inc-only), and nonseparated (nonsep) cells. Treatment groups that were treated with HEPES buffer with or without trypsin were not statistically different from each other and so were combined for presentation here. Bars represent the mean ± SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 6

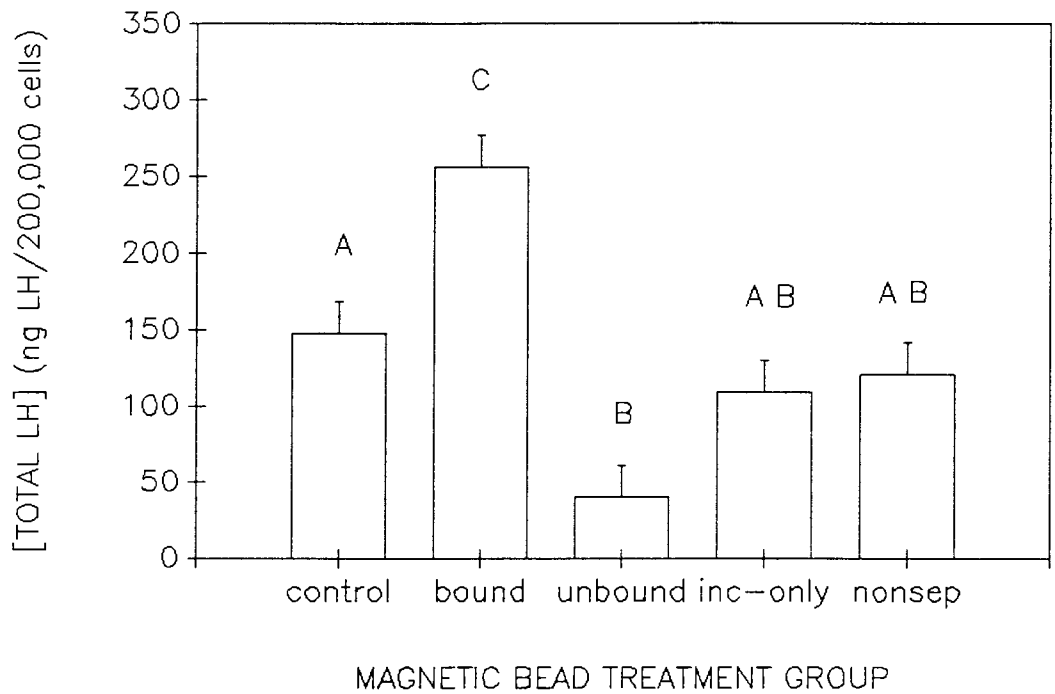

[TOTAL LH] (ng LH/200,000 cells)
350
300
250
200
150
100
50
0
A
C
B
A B
A B
control
bound
unbound
inc-only
nonsep
MAGNETIC BEAD TREATMENT GROUP

Figure 7 : Experiment 1-Medium FSH

I measured FSH via RIA in medium samples of the bound, unbound, incubated-only (inc-only), and non-separated (nonsep) cells. Treatment groups that were treated with HEPES buffer with or without trypsin were not significantly different from each other and so were combined for presentation here. Bars represent the mean ± SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 7

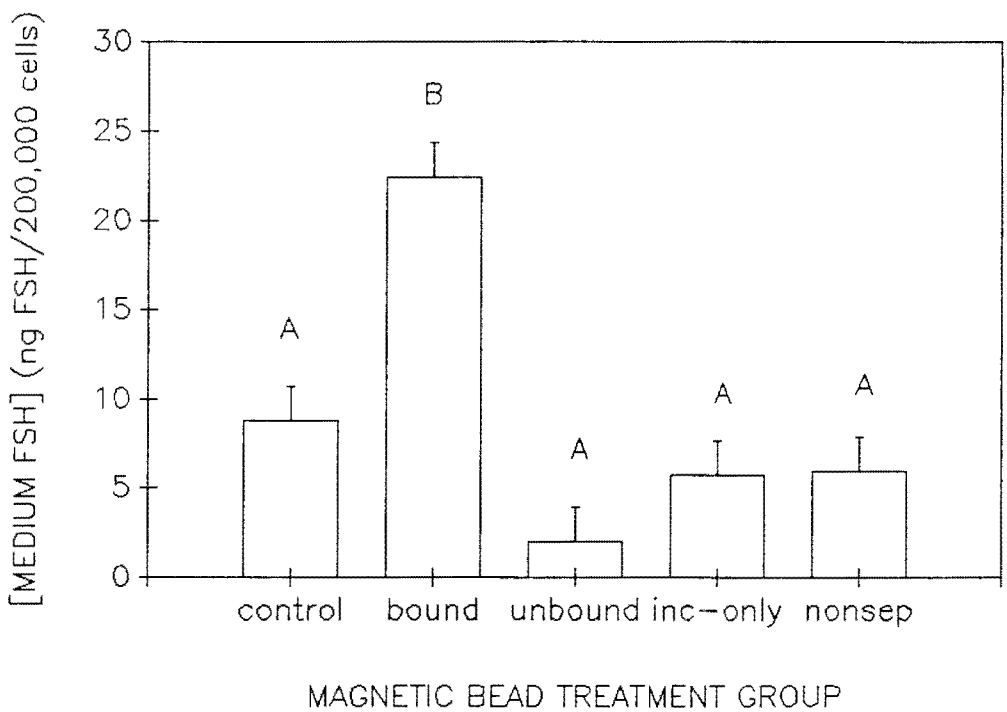

[MEDIUM FSH] (ng FSH/200,000 cells)
30
25
20
15
10
5
0
A
B
A
A
A
control
bound
unbound
inc-only
nonsep
MAGNETIC BEAD TREATMENT GROUP

Figure 8 : Experiment 1-Cellular FSH

I measured FSH via RIA in cell lysates of the bound, unbound, incubated-only (inc-only), and nonseparated (nonsep) cells. Treatment groups that were treated with HEPES buffer with or without trypsin were not significantly different from each other and so were combined for presentation here. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 8

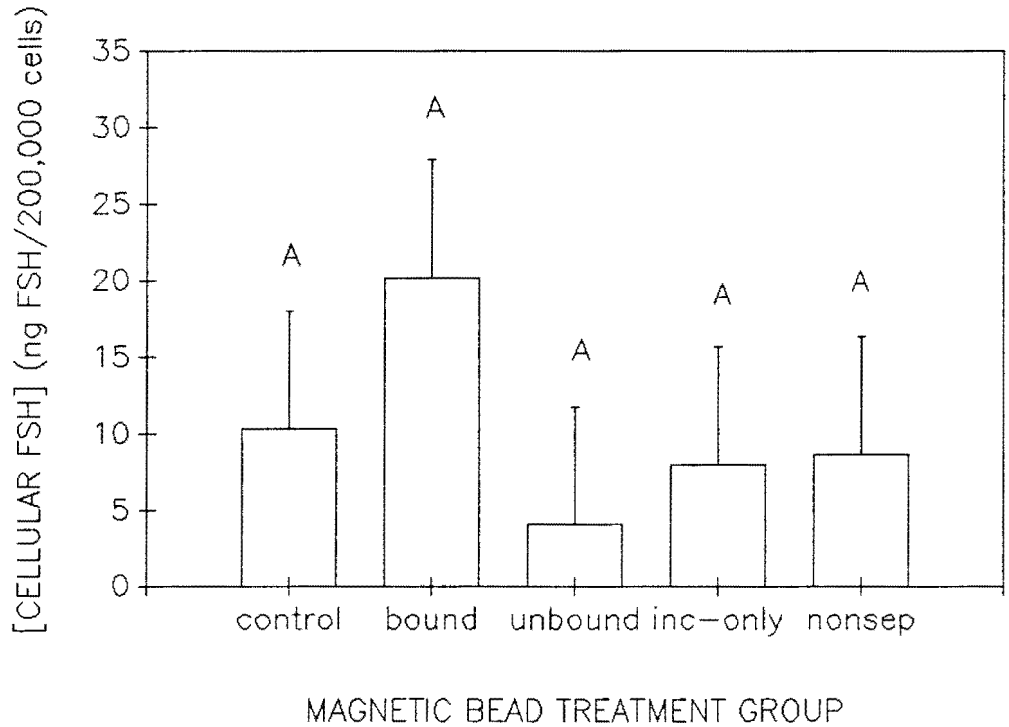

[CELLULAR FSH] (ng FSH/200,000 cells)
35
30
25
20
15
10
5
0
A
A
A
A
A
control
bound
unbound
inc-only
nonsep
MAGNETIC BEAD TREATMENT GROUP

Figure 9 : Experiment 1-Total FSH

I calculated total FSH by adding medium and cellular FSH in the bound, unbound, incubated-only (inc-only), and nonseparated (nonsep) cells. Treatment groups that were treated with HEPES buffer with or without trypsin were not significantly different from each other and so were combined for presentation here. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 9

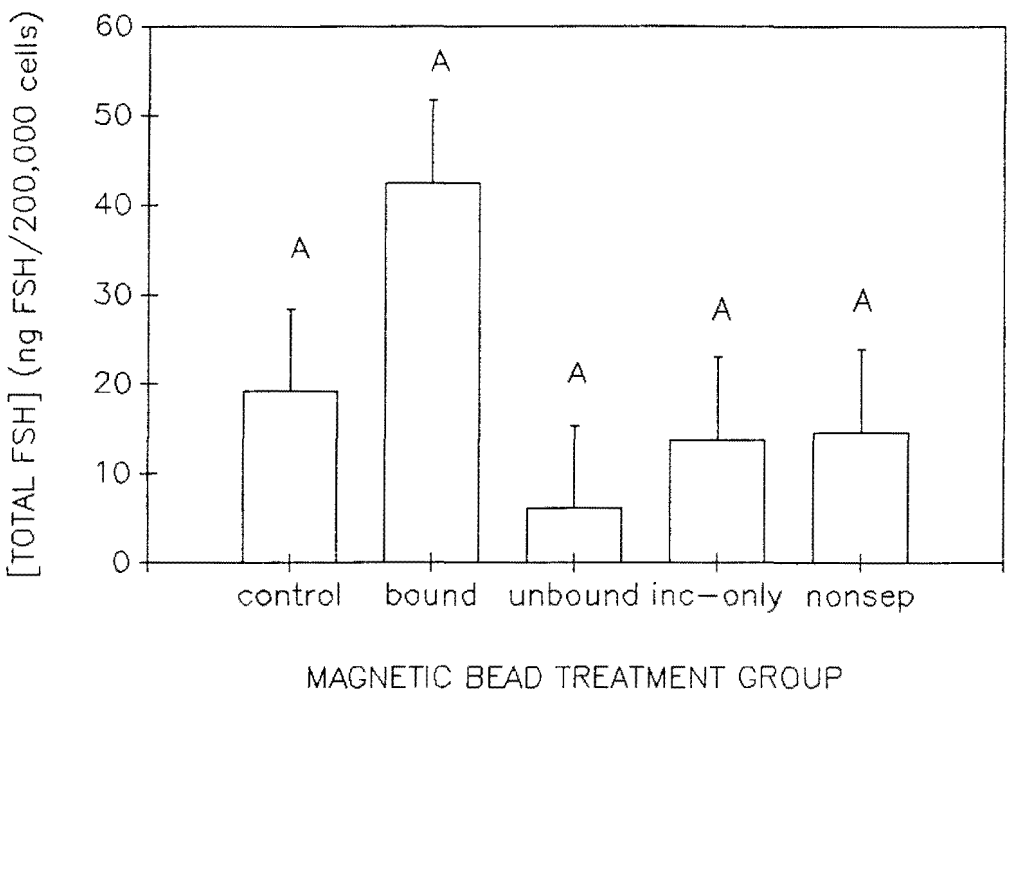

[TOTAL FSH] (ng FSH/200,000 cells)
60
50
40
30
20
10
0
A
A
A
A
A
control
bound
unbound
inc-only
nonsep
MAGNETIC BEAD TREATMENT GROUP

Figure 10 : Experiment 2-medium LH

I measured LH via RIA in media of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean ± SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Groups with different letters are significantly different from each other as determined by Tukey's test.

Figure 10

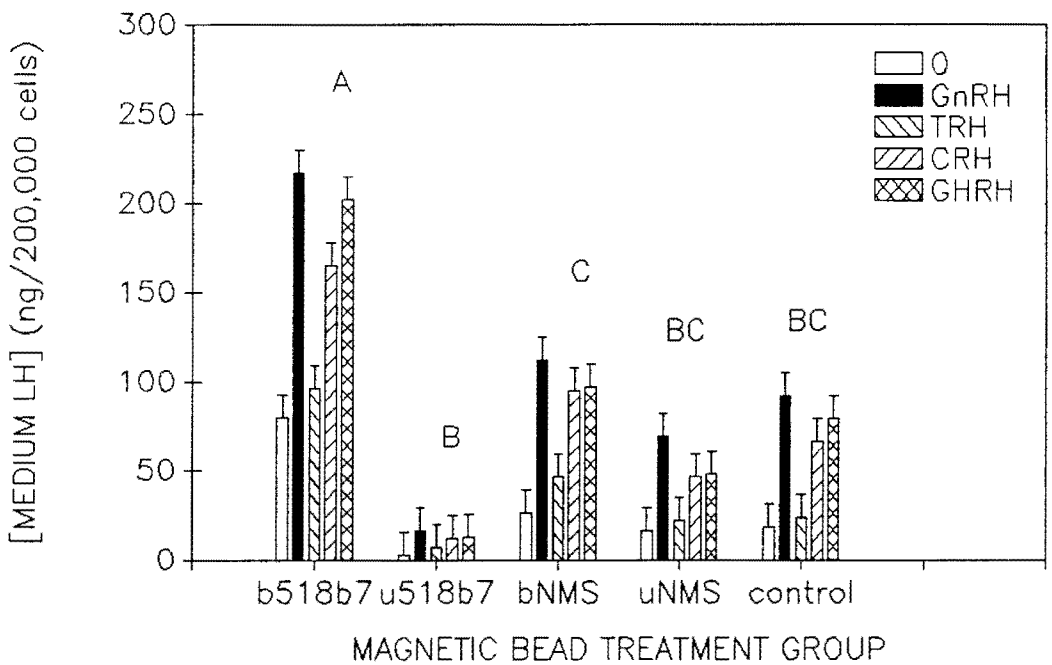

[MEDIUM LH] (ng/200,000 cells)
300
250
200
150
100
50
0
A
B
C
BC
BC
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 11 : Experiment 2-cellular LH

I measured LH via RIA in lysates of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 11

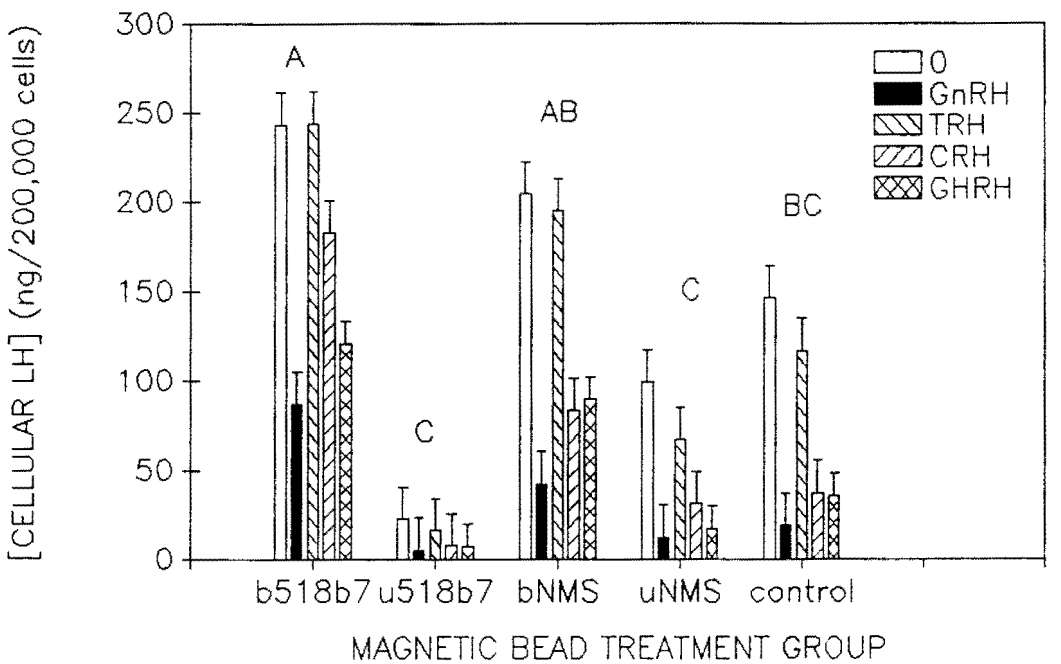

[CELLULAR LH] (ng/200,000 cells)
300
250
200
150
100
50
0
A
C
AB
C
BC
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 12 : Experiment 2-total LH

I calculated total LH by adding the medium and cellular values for the cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. Bars represent the mean ± SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 12

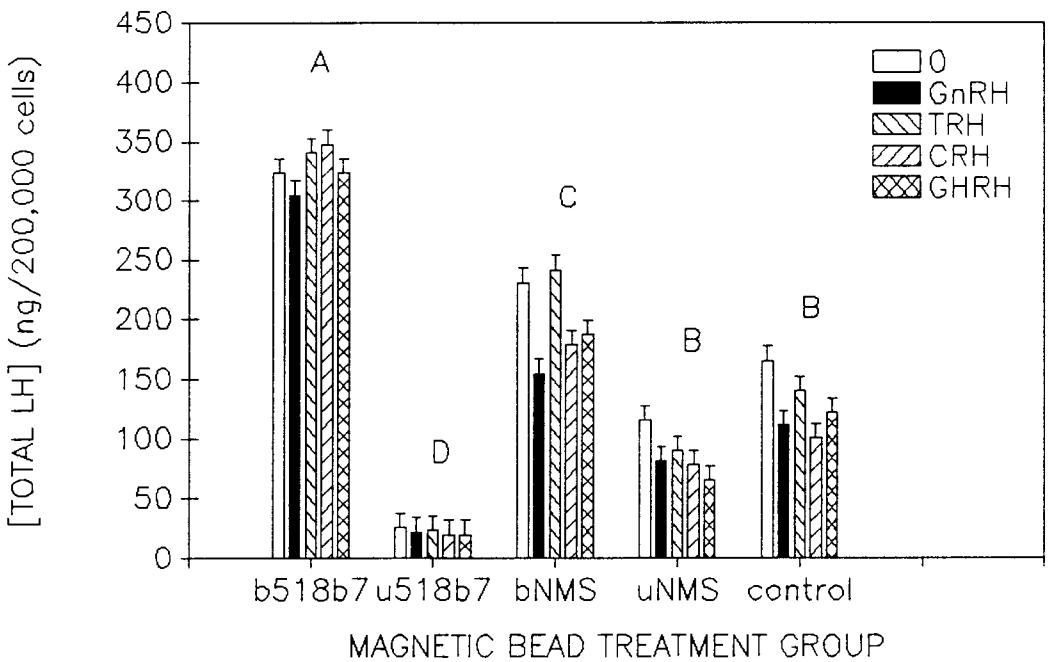

[TOTAL LH] (ng/200,000 cells)
450
400
350
300
250
200
150
100
50
0
A
D
C
B
B
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 13 : Experiment 2-medium FSH

I measured FSH via RIA in media of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 13

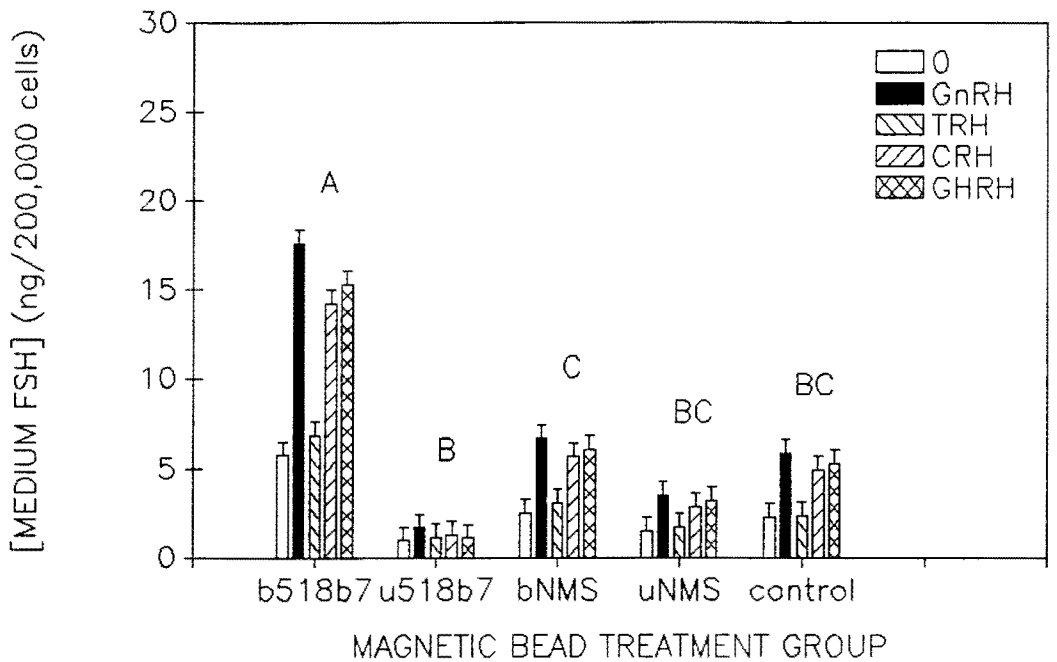

[MEDIUM FSH] (ng/200,000 cells)
30
25
20
15
10
5
0
A
B
C
BC
BC
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 14 : Experiment 2-cellular FSH

I measured FSH via RIA in lysates of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 14

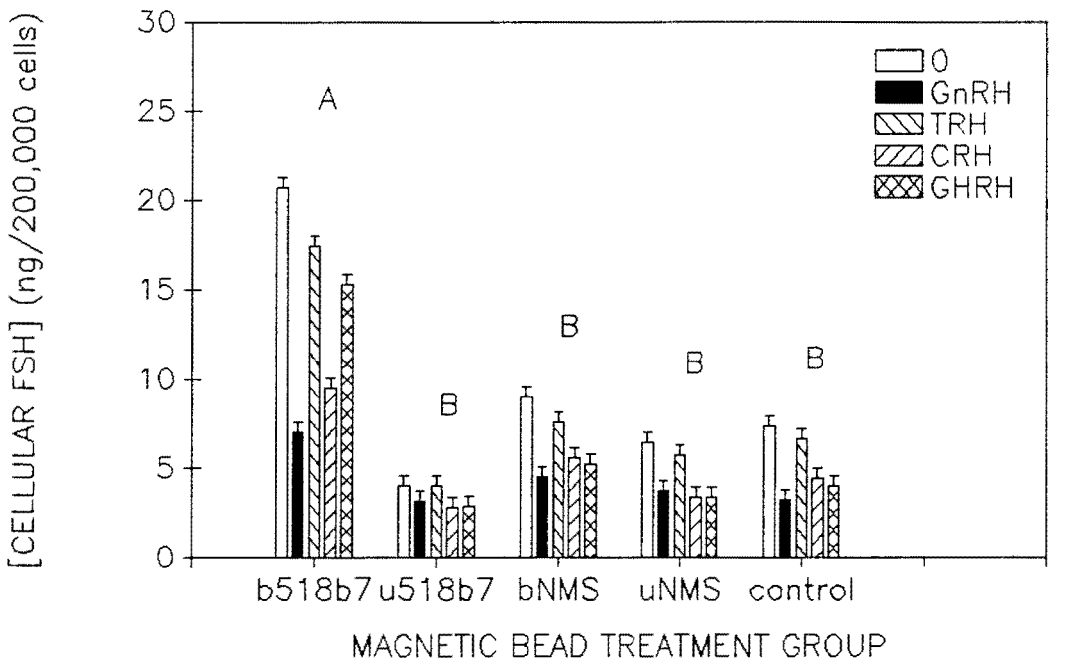

[CELLULAR FSH] (ng/200,000 cells)
30
25
20
15
10
5
0
A
B
B
B
B
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 15 : Experiment 2-total FSH

I calculated total FSH by adding the medium and cellular values for the cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. Bars represent the mean ± SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 15

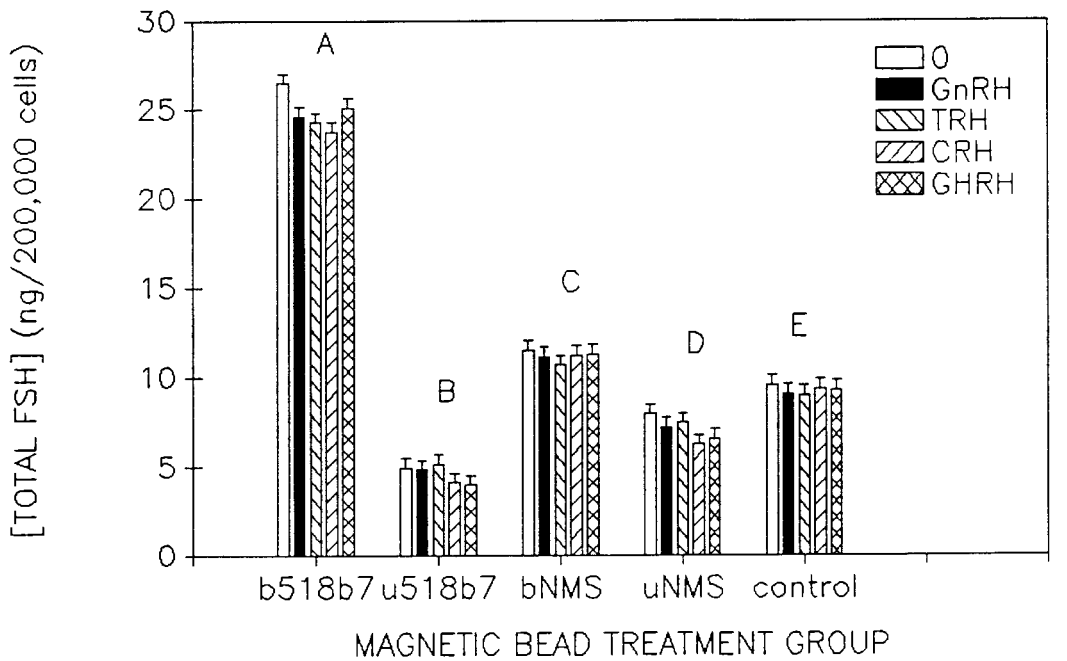

[TOTAL FSH] (ng/200,000 cells)
30
25
20
15
10
5
0
A
B
C
D
E
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 16 : Experiment 2-medium PRL

I measured PRL via RIA in media of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance.

Figure 16

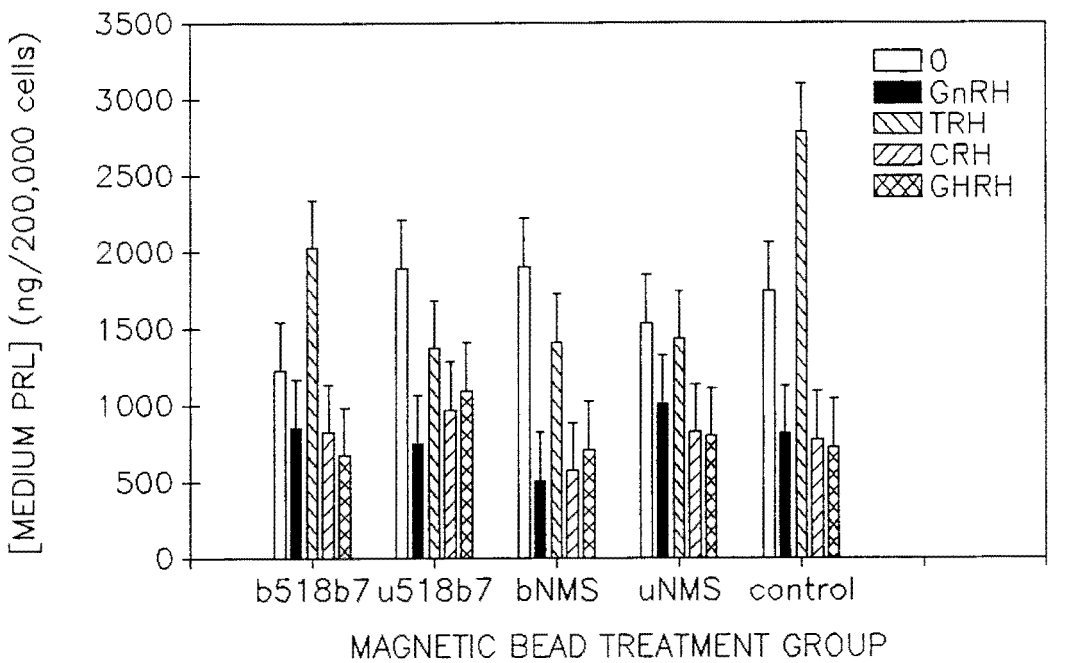

[MEDIUM PRL] (ng/200,000 cells)
3500
3000
2500
2000
1500
1000
500
0
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 17 : Experiment 2-cellular PRL

I measured PRL via RIA in lysates of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance.

Figure 17

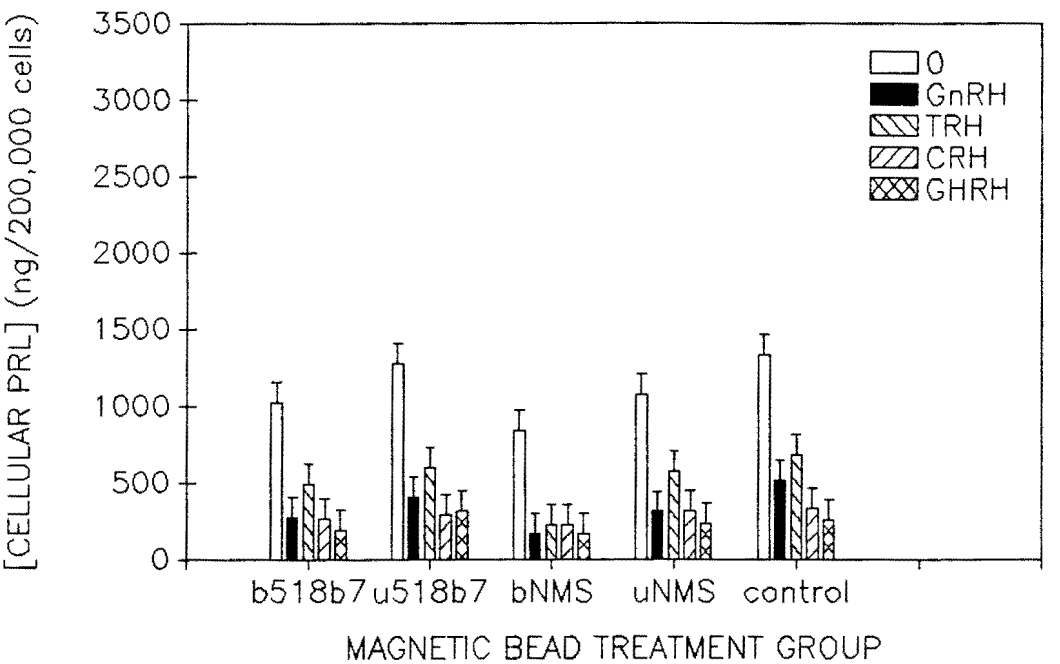

[CELLULAR PRL] (ng/200,000 cells)
3500
3000
2500
2000
1500
1000
500
0
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 18 : Experiment 2-total PRL

I calculated total PRL by adding the medium and cellular values for the cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance.

Figure 18

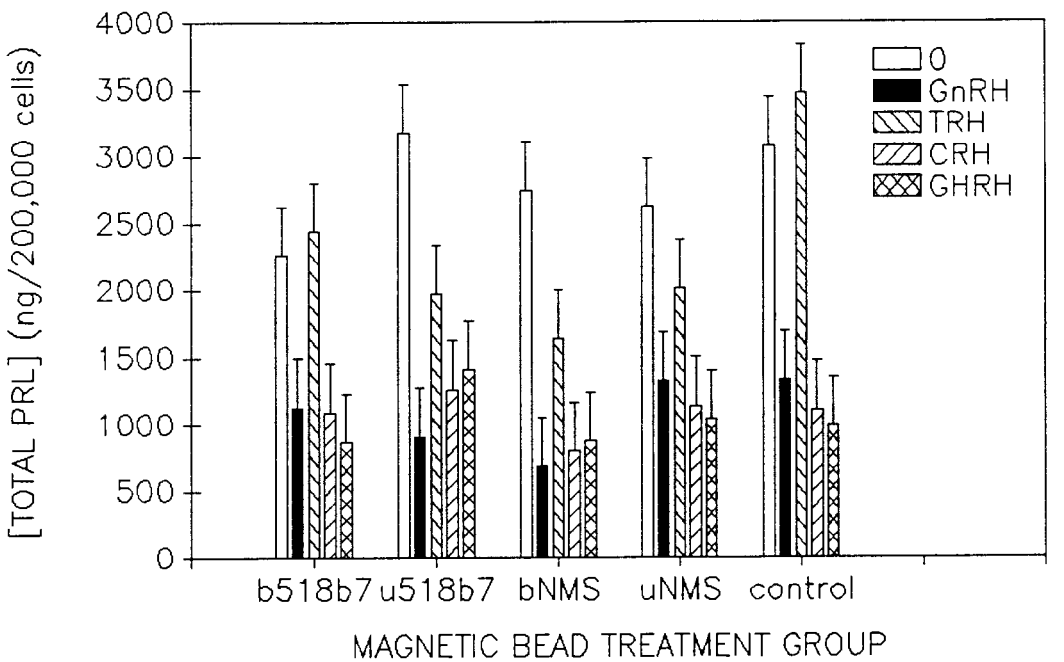

4000
3500
3000
2500
2000
1500
1000
500
0
[TOTAL PRL] (ng/200,000 cells)
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 19 : Experiment 2-medium TSH

I measured TSH via RIA in media of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance.

Figure 19

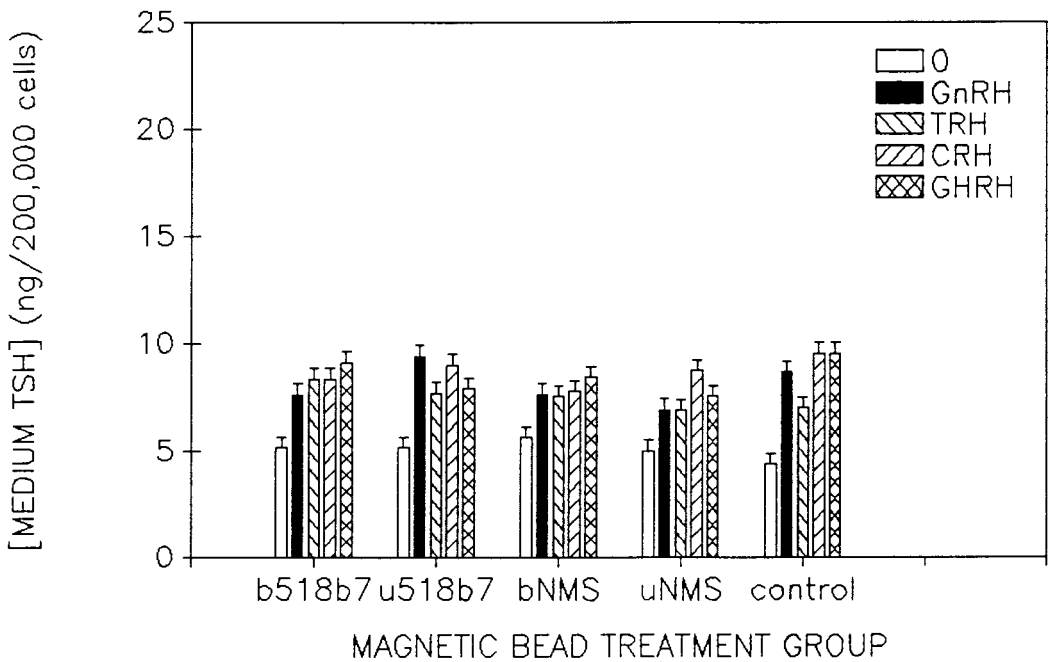

[MEDIUM TSH] (ng/200,000 cells)
25
20
15
10
5
0
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 20 : Experiment 2-cellular TSH

I measured TSH via RIA in lysates of cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. I treated some cells from each group with GnRH, TRH, CRH, GHRH, or no releasing factor. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance.

Figure 20

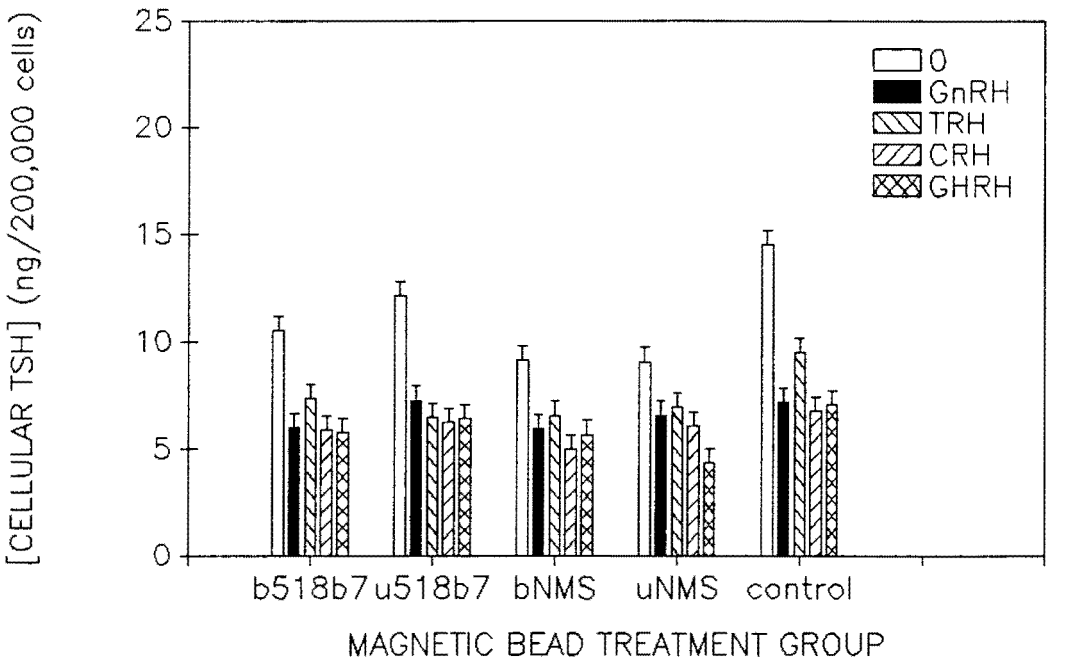
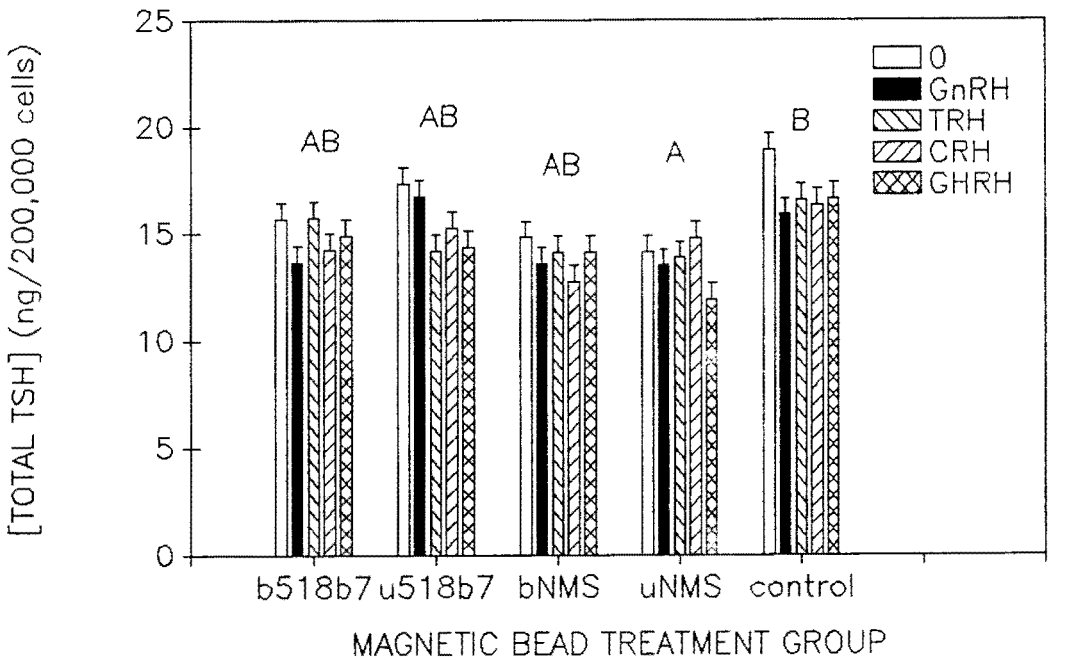

[CELLULAR TSH] (ng/200,000 cells)
25
20
15
10
5
0
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Figure 21 : Experiment 2-total TSH

I calculated total TSH by adding the medium and cellular values for the cells bound to 518b7db (b518b7), not bound to 518b7db (u518b7), bound to NMSdb (bNMS), not bound to NMSdb (uNMS), and control cells. Bars represent the mean $\pm$ SEM of three experiments, with the SEM having been determined from the error mean square of the analysis of variance. Bars with different letters are significantly different from each other as determined by Tukey's test.

Figure 21


[TOTAL TSH] (ng/200,000 cells)
25
20
15
10
5
0
AB
AB
AB
A
B
0
GnRH
TRH
CRH
GHRH
b518b7
u518b7
bNMS
uNMS
control
MAGNETIC BEAD TREATMENT GROUP

Discussion

The purpose of this thesis project was to develop a new method to separate LH-cells from a mixed population of rat anterior pituitary cells with the use of anti-LH antibody-coated magnetic beads (518b7db). Once this method was developed, it was used to support the hypothesis that LH and FSH are both contained in and secreted from the same pituitary cell. Before I performed the separation experiments, I first performed specificity checks 1 and 2 so that I could be certain that 518b7db were specific for LH. Through specificity check 1, I showed that 518b7db were specific for LH because binding of ^{125}I-LH to 518b7db increased as the concentration of 518b7db increased, whereas there was no specific binding of ^{125}I-FSH to 518b7db. Through specificity check 2, I showed that 518b7db were specific for LH because unlabeled LH competed with ^{125}I-LH for binding to 518b7db, whereas unlabeled FSH did not compete with ^{125}I-LH for binding to 518b7db. From these specificity checks, I concluded that 518b7db were specific for LH and therefore were suitable for use in the separation experiments. In addition, I ran a time course study to determine how long the anti-LH antibody used in these studies would remain attached to

the magnetic beads. From this study, I determined that the anti-LH antibody would remain attached to the magnetic beads for a period of at least 5 weeks. Therefore, I determined that I could use 518b7db for a period of at least 5 weeks because anti-LH antibody did not dissociate from the magnetic beads during this time period.

The second step of this thesis project was to use 518b7db to perform experiment 1. During experiment 1, the negative separation procedure was used and inc-only and nonsep cells served as controls for this procedure. The LH data from experiment 1 suggests that I have shown that, with the use of 518b7db, it is possible to obtain an enriched fraction of viable LH-secreting anterior pituitary cells from a mixed pituitary cell population. Cells that were attached to the 518b7db secreted and contained more LH than unbound, inc-only, nonsep, and control cells.

The increased amount of LH contained and secreted by cells bound to 518b7db is due to specificity of the separation procedure and not due to experimental artifacts because inc-only and nonsep cells did not differ from control cells. For example, the inc-only cells served as a control for the long incubation period used during the separation procedure. Since the amount of LH secreted by and contained in these cells was not significantly different from control cells, I have shown that pituitary

cells remain viable during the incubation procedure and that incubation did not cause a change in the measured amount of LH secreted by or contained in a pituitary cell.

The non-sep cells served as a control for the presence of 518b7db in the separation procedure. Since the amount of LH secreted by and contained in these cells did not differ from control cells, I have shown that the presence of 518b7db did not cause a change in the measured amount of LH secreted by or contained in a pituitary cell.

In addition to measuring LH secretion and content in experiment 1, I also measured FSH secretion and content. The FSH data obtained show that the amount of FSH secreted by b518b7 cells is greater than the amount of FSH secreted by control, unbound, inc-only, and nonsep cells. These data suggest that cells bound to 518b7db secrete an increased amount of FSH as well as an increased amount of LH compared to all other magnetic bead treatment groups. When I compared the ratios of LH to FSH in the various magnetic bead treatment groups, I found that the ratios of LH to FSH did not differ between the cells bound to 518b7db and control cells. This indicates that I have only separated cells that secrete both LH and FSH from the mixed cell population and that I did not separate any cells that secrete only LH because the ratio of LH to FSH was not increased in cells bound to 518b7db compared to control. These data were not unexpected because there is

evidence from immunocytochemistry that most gonadotropes secrete both LH and FSH and only a small population of gonadotropes secrete only LH or FSH.[6,7] Thus, it would be very difficult to remove the few cells that secrete only LH from a mixed cell population unless anti-FSH dynabeads were available.

The LH data from experiment 2 also show that the amount of LH contained and secreted by b518b7 cells was increased compared to the u518b7, bNMS, uNMS, and control groups. These data again suggest that I have obtained an enriched fraction of LH-cells.

I am certain that the increased LH secretion and content in b518b7 cells during experiment 2 was due to the specificity of the 518b7db binding to LH-secreting cells because NMSdb served as a control for nonspecific binding. NMSdb nonspecifically bind to LH-secreting cells as well as all other cell types present because cells do not contain a specific receptor for NMS. The nonspecificity of NMSdb was shown in specificity checks 1 and 2 through the dose-inhibition and dose-response curves. The binding of ^{125}I-LH to NMSdb did not increase as the concentration of NMSdb increased and ^{125}I-LH and nonradiolabeled LH did not compete for binding to NMSdb. Therefore, since the amount of LH secreted and contained by cells nonspecifically bound to NMSdb was less than the amount of LH secreted and contained by b518b7 cells, I have shown the

separation procedure specifically removes LH-cells.

During experiment 2, I also measured the amounts of FSH, TSH, and PRL secreted and contained by the different treatment groups. B518b7 cells contained and secreted an increased amount of FSH as compared to u518b7, bNMS, uNMS, and control cells. These data again suggest that I have removed pituitary cells that contain and secrete both FSH as well as LH. When I compared the ratio of LH to FSH in the different magnetic bead treatment groups, I found that there was no difference between the ratios of LH to FSH in cells bound to 518b7db and control cells. Again, this suggests that I have only removed cells that secrete and contain LH and FSH. These data support my hypothesis as well as the aforementioned results obtained with immunocytochemistry.[6,7]

The PRL and TSH data obtained from experiment 2 show that there are no significant differences between the amounts of PRL or TSH secreted by or contained in the b518b7, u518b7, bNMS, uNMS, and control cells. These data were expected because there is no evidence in the literature that supports the hypothesis that LH gonadotropes also contain and secrete PRL or TSH. I did not reduce the amount of PRL or TSH secreted by or contained in b518b7 cells as compared to controls because I used a negative selection separation procedure so that I could obtain a large population of LH-secreting cells. During negative

selection, a large number of magnetic beads per cell is used to remove most cells secreting LH from a mixed anterior pituitary population. It is also possible that, because of the large number of magnetic beads used, some non-LH-secreting cells are also removed from the mixed population. In the future, it is possible to perform positive selection experiments which use a fewer number of magnetic beads per cell during the separation procedure. This use of fewer beads would insure the removal of only a few pure gonadotropes from a mixed cell population and avoid the removal of non-LH-secreting cells.

During experiment 2, I also treated some cells from each treatment group with GnRH, TRH, GHRH, CRH, or no releasing factor. This procedure was performed to show that the b518b7 cells were gonadotropes. For example, b518b7 cells should respond only to GnRH by increasing LH and FSH secretion and not to any other releasing factors because there is no evidence in the literature that TRH, GHRH, or CRH affect LH and FSH cell content and secretion. In addition, control cells and unbound cells should respond to TRH, CRH, and GHRH because they contain TSH-, PRL-, ACTH-, and GH-cells.[41,42,43] The results that I obtained were unexpected and inconsistent. For example, I observed that in some cases CRH, TRH, and GHRH caused stimulation of LH and FSH. I also observed in some cases that GnRH affects PRL and TSH release and content. These

results suggest that further studies need to be done to determine exactly what effects various releasing factors have on LH, FSH, TSH, and PRL secretion and content. It is possible, however, that these inconsistent and unexpected data are due to cross-contamination of the releasing factors used during experiment 2.

All of the aforementioned data show that I have developed a new method which can be used to obtain an enriched fraction of LH gonadotropes. The use of this method enabled me to obtain a 3-fold purification of LH-cells. Although it is possible to obtain a 6 to 8-fold purification of LH-cells with the use of a flourescence-activated cell-sorter, centrifugal elutriation, and velocity sedimentation at unit gravity, this method can still be more useful than other techniques. This method is better than older techniques such as velocity sedimentation at unit gravity, centrifugal elutriation, fluorescence-activated cell-sorting, and forward angle and perpendicular light scatter because it is far cheaper since it does not require a fluorescence-activated cell-sorter, it allows a large number of cells to be separated at once, and virtually 100% of these cells remain viable. This separation technique can also be used in place of the reverse hemolytic plaque assay which does not prove useful for the study of FSH since it is difficult to obtain anti-FSH antibodies suitable for use with immunocytochemical

techniques. Instead, this technique makes it possible to use anti-FSH antibody coated magnetic beads to obtain a pure population of FSH gonadotropes. In the future, it will also be possible to use this method to remove any cell type from a mixed cell population provided an antibody to the particular cell type is available.

CONCLUSIONS

1. I have developed a fast, reliable, and inexpensive method to selectively remove gonadotropes that contain both LH and FSH from a mixed rat anterior pituitary cell population.

2. I have used this method to support the hypothesis that most of the LH that is secreted from the anterior pituitary is secreted from cells that contain both LH and FSH.

3. This new method can be used to remove any cell type from a mixed cell population provided an antibody to the particular cell type is available.

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APPROVAL SHEET

The thesis submitted by Jodi Flaws has been read and approved by the following committee:

Dr. Diane Suter, Director
Assistant Professor, Biology, Loyola University

Dr. Howard Laten
Professor, Biology, Loyola University

Dr. Frederick Wezeman
Professor, Biology, Loyola University

The final copies have been examined by the director of the thesis and the signiture which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Masters of Science.

May 4 1990
Date

Diane Suter
Advisor