

SOURCEBOOK OF LABORATORY ACTIVITIES IN PHYSIOLOGY

Altering rat sexual behavior to teach hormonal regulation of brain imprinting

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Submitted 13 June 2019; accepted in final form 1 August 2019

Geisert RD, Schmelzle AL, Smith MF, Green JA. Altering rat sexual behavior to teach hormonal regulation of brain imprinting. *Adv Physiol Educ* 43: 458–466, 2019; doi:10.1152/advan.00081.2019.—In this teaching laboratory, students design and perform an experiment to determine estrogen's role in imprinting the brain of neonatal rats to express either male or female sexual behavior. A discussion question is provided before the laboratory exercise in which each student is asked to search the literature and provide written answers to questions and to formulate an experiment to test the role of estrogen in imprinting the mating behavior of male and female rats. Students discuss their answers to the questions in laboratory with the instructor and design an experiment to test their hypothesis. In male rats, testosterone is converted by aromatase expressed by neurons in the brain to estrogen. Production of estrogen in the brain of neonatal rats imprints mating behavior in males, where a lack of estrogen action in the brain imprints female sexual behavior. The model involves administering exogenous testosterone to imprint male behavior in female pups or administration of an aromatase inhibitor (letrozole) or an estrogen receptor antagonist (ICI 182,780) to imprint female sexual behavior in male pups. In the model, litters of neonatal pups are treated with either carrier (control), testosterone propionate, aromatase inhibitor (letrozole), or an estrogen receptor antagonist (ICI 182,780) postnatally on *days 1* and *3*. Alteration of mating behavior is evaluated through the numbers of males and females that breed and establish pregnancy. This is a very simple protocol that provides an excellent experiment for student discussion on the effects of hormone action on imprinting brain sexual behavior.

aromatase inhibitor; estrogen; estrogen receptor antagonist; imprinting; rat; sexual behavior; testosterone

INTRODUCTION

Objectives and Overview

The present laboratory exercise makes use of the fact that several physiological phenomena and behavioral responses to endocrine stimulation are disproportionately sex specific. This is clearly evident in reproductive sexual behavior of males and females. The process of masculinization/defeminization of the developing neonatal brain involves both genetic and hormonal regulation. Alteration of the normal hormonal exposure during the time when imprinting of sexual behavior is taking place in the rat can lead to feminized male and masculinized female behavior in adults. This laboratory exercise will demonstrate to the students how researchers manipulate biological mechanisms to better understand them. Furthermore, it helps students understand the sensitivity and plas-

ticity of the neonatal brain to imprinting by steroid hormones released during pregnancy and the potential effect of environmental endocrine disruptors, including various pollutants (7).

Background

The biological sex of most mammals is regulated by genetics (presence or absence of a Y chromosome), gonad development, establishment of male or female internal and external reproductive anatomy, and release of sex hormones. Although differences in the brain of male and females exist beyond reproduction, specific outcomes arising from changes in reproductive behaviors are clearly evident and easy to observe. Sexual dimorphism of the rat brain is established postnatally the first week after birth (1). The native brain sex of neonatal offspring of mammals is inherently programmed to be feminine (default sex), unless neuron-specific changes occur in the brain through stimulation by testicular androgens (testosterone) to express male sexual breeding behavior.

The role of testicular androgens in masculinizing sex hormone secretion, and ultimately sex-specific behavior, was first recognized by Pfeiffer (9). Later, research by Barraclough (1) demonstrated that treatment of neonatal female pups with testosterone propionate masculinized hormonal secretion and sexual behavior. Research indicated that the medial preoptic area in the hypothalamus of the treated females was inhibited from stimulating ovulation in the females. Importantly, castration of male rats on *day 3* postnatally resulted in endocrine patterns of luteinizing hormone (LH) secretion, similar to the surge release of LH to induce ovulation in intact females (5). These findings demonstrated that removal of testes (androgen source) resulted in formation of the “default” feminized neural network in the male (3).

The previous information indicates that the neonatal brain is inherently female but becomes masculinized through exposure to testicular androgens. This led to the conclusion that androgens (e.g., testosterone) directly stimulate imprinting of the neonatal brain to elicit male sexual behavior. However, testosterone is actually indirectly involved with imprinting behavior, since estrogen is the actual steroid that triggers formation of the neural cell network and receptivity for masculinization of the brain (4). Testosterone freely crosses the blood-brain barrier and is converted to estrogen through aromatase expressed by neurons in the brain. Thus estrogen coupling to neural estrogen receptors (ER) induces the neural organization that leads to masculine behaviors and hormone secretion patterns. The role of estrogen in imprinting a masculine brain is also supported by the ability to block masculine behavior in males after

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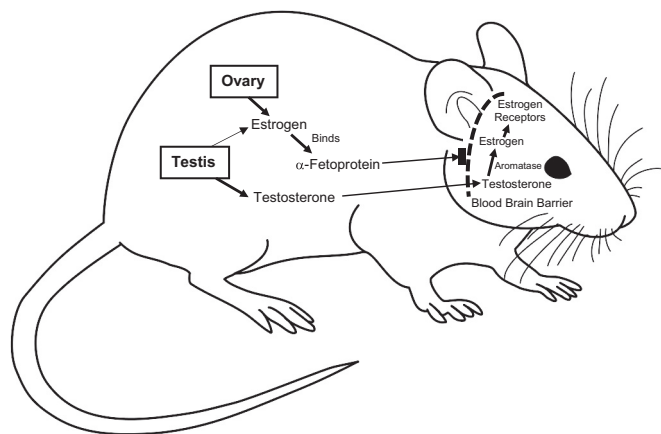


Fig. 1. Model of the role of plasma testosterone passing through the blood-brain barrier and being converted to estrogen in the brain to imprint male sexual behavior postnatally. In the female, estrogen binds to α -fetoprotein, which prevents passage through the blood-brain barrier, resulting in brain imprinting for female sexual behavior.

treating the neonates with anti-estrogens (2) or an aromatase inhibitor (6).

The content of estrogen in newborn pups is quite high in both sexes (8), which seems counterintuitive, since estrogen is essential for masculinizing the brain. If estrogen is involved with sex brain differentiation in males, then how is the brain of female rats not masculinized in the presence of high concentrations of placental estrogens? This inconsistency can be addressed through the presence of α -fetoprotein in the neonatal blood. The livers of both male and female fetuses synthesize α -fetoprotein, which has a high binding affinity for estrogen. Indeed, its affinity for estrogen is greater than ESR1 (the genomic ER). Furthermore, α -fetoprotein does not bind androgens (4). Therefore, in males, although α -fetoprotein complexes with estrogen and minimizes transfer across the blood-brain barrier in both male and females, testosterone from the testes freely moves into the brain and is converted into estrogen; thus inducing masculinization through binding to ER present in neurons (Fig. 1). In the absence of testosterone in the female (default sex), neurons are programmed to exhibit feminine behavior and the preovulatory gonadotropin surge required for ovulation.

The classical models to demonstrate sexual differentiation of the brain are to either administer testosterone to neonatal female rats (which causes masculinization), or castrate neonatal males to induce the “default” sex, which leads to typical feminine mating behaviors. To establish a rat sexual differentiation model, we established a protocol that utilizes testosterone propionate to masculinize sexual behavior of female neo-

nates and either an ER antagonist (ICI 182,780), which inhibits estrogen action through ER, or an aromatase inhibitor (letrozole) to feminize sexual behavior of male neonates. Consequently, the inhibition of masculinization can be accomplished without the need for castration of neonatal males, thus providing intact adult males to test fertility (as an outcome of normal or altered sex-specific behaviors).

In this exercise, each litter is administered one of several treatments: corn oil, testosterone propionate, ICI 182,780 (ER antagonist), or letrozole (aromatase inhibitor). The treatments are administered on postnatal *days 1 and 3*. The injection of testosterone propionate will masculinize the females in the litter, whereas letrozole blocks the ability of testosterone to be converted to estrogen in the brain. In theory, ICI 182,780 (ER antagonist) should prevent masculinization of the males, but it is actually ineffective. Because ICI 182,780 is very similar to estrogen (Fig. 2); it binds to α -fetoprotein, which prevents the ER antagonist from entering the brain (4). This is a simple protocol that is effective and provides an excellent experiment for students to comprehend hormone action and the use of agonists and antagonists on altering the sexual neural network in the rat brain. Students will not directly observe the animals’ sexual activity, which requires the ability to video record copulatory behavior. This teaching exercise allows the students to indirectly evaluate breeding behavior by monitoring the animals for copulatory plugs, counting implantation sites, and recording the number of pregnancies established at the end of the experiment. Feminized males may show a lack of interest in copulating, which will be revealed through lack of copulatory plugs during the breeding period and the absence of implantation sites at the termination of the experiment. Masculinized females may allow males to mate with them over several days, but they will usually not contain implantation sites due to the absence of an LH surge and a failure to ovulate.

The laboratory was designed to engage the students in designing the experiment following a lecture on hormone regulation of sexual differentiation and discussion among their laboratory group. Students can be involved with administering treatments postnatally to the pups, if the instructor wants to provide them an opportunity to be more involved in understanding the importance of treatments and timing of injections to alter sexual behavior and mating of mature females and males. Evidence of mating activity is determined based on the establishment of pregnancy when the rats have reached sexual maturity at ~60 days of age and beyond. Students can be involved with plug checking females (i.e., evidence a female has been mated) during breeding, which requires ~30–40 min over 5–7 days, and data collection from the males and females, which can be accomplished in a 2- to 3-h laboratory.

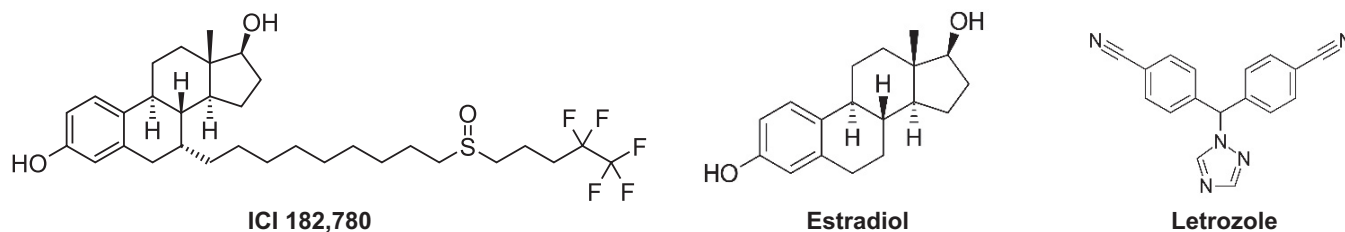


Fig. 2. Structural models for ICI 182,780, estradiol, and letrozole.

Learning Objectives

The overall objective of the laboratory exercise is designed to provide a hands-on learning experience in the role of steroids in establishing brain sexual behavior in mammals.

- A. Appraise the biological effects of steroid hormones on altering brain imprinting for mating behavior in rats.
- B. Demonstrate the actions of estrogen, testosterone, receptor antagonists, and aromatase inhibitors on regulation of brain imprinting for sexual behavior in rats.
- C. Interpret how environmental pollutants and hormone mimics (e.g., endocrine disruptors) may alter sexual behavior and mating in mammals.
- D. Construct an experiment to effectively test the hypothesis that steroids can alter sexual breeding behavior of neonatal rats (e.g., positive control, negative control, treatment groups, data collection, analysis).

Activity Level

This activity is suitable for an undergraduate course in biology, premedicine, wild life, physiology, and animal science. It can also be tailored for instruction of graduate students in behavior, reproductive biology, physiology, and psychology. We perform this laboratory exercise in our undergraduate junior/senior honors Physiology of Reproduction course.

Prerequisite Student Knowledge or Skills

If the students will participate in the hands-on administration of treatments or the evaluation of mating (e.g., looking for copulatory plugs), the students should receive basic instruction about safe handling of rats, giving subcutaneous injections, and fundamental background in animal welfare. Knowledge of physiology, endocrinology, reproductive biology, statistics, and animal behavior would greatly strengthen student comprehension of the laboratory exercise.

The honors Physiology of Reproduction course, in which this laboratory exercise is employed, is a combined lecture/laboratory class. The first lectures in the course address genetic and physiological regulation of sexual differentiation, gonad development, development of male or female internal and external reproductive anatomy, release of sex hormones, and brain imprinting for sexual behavior. Before the lecture on imprinting sexual behavior of the brain, students are provided two review papers (4, 5) on brain sexual dimorphism to obtain a basic background for developing the experiment for the second week of the course. This allows students to understand what treatments they will apply to the neonatal rats and the timing for the treatments. It is important that students be aware of the structural differences (Fig. 2) in letrozole (aromatase inhibitor) and ICI 182,780 (ER antagonist) to understand results of the experiment based on their knowledge of the blood-brain barrier. Throughout the semester, students should gain an understanding of basic reproductive anatomy, hormones, and regulation of the estrous cycle and pregnancy. These topics provide the background for students to undertake collection of the data following breeding for the laboratory exercise. Students are involved with the breeding of female rats, so they need to be trained on proper animal handling and restraint to perform the checks for vaginal plugs and to correctly administer subcutaneous injections to the neonates (if

that aspect is included as part of the overall laboratory experience). The students working with the animals are instructed and overseen by the instructors or by very experienced graduate teaching assistants or research specialists. All animal procedures and animal worker qualifications are reviewed and approved by the institutional animal care and use committee (IACUC).

Time Required

Pregnant female rats can either be purchased or bred, if a rat colony is available. However, the females need to give birth (i.e., pup) during the 2nd to 3rd week (of a typical 15-wk semester) to be mature enough to breed and collect data by the 14th week of the semester. Once the females give birth, it takes ~15 min for the students to give the subcutaneous injections on the afternoon of birth and 48 h postbirth. Not all females will pup on the same day, so treatments may be across 2–5 days. Students can be involved with sorting males and females into cages at 28 days of age, which requires <1 h. On reaching sexual maturity at 56 days of age, sorting treated males and females into cages for breeding requires ~1 h. Plug checking females can be accomplished in <1 h in the morning for 5–7 days. The time to set up mating of females until dissection of the reproductive tracts for the laboratory exercise takes 15–21 days, depending on when the instructor wishes to evaluate the latest day of pregnancy. The laboratory exercise to weigh rats; dissect the uterus and visualize implantation sites and the placental membranes of the females; weigh the testes, epididymis, and accessory glands of the males; and record data takes ~2 h.

Alternate Activity to Shorten Exercise

The experiment requires ~13–14 wk to complete, which may not be suitable to courses held during a quarter-semester system. The activity could be modified by the instructor to pup females and administer treatments to the neonatal pups before the start of the course. This would allow litters to born slightly further apart, if they are not available to pup within 1–5 days. Also, the time to breed males and females can be adjusted to when it is best suited for the laboratory during the course. The instructor can generate animals representing each treatment group and then allow the students to participate in plug checks during the mating period. Thus the time needed to conduct the exercise would encompass only 3–4 wk.

METHODS

Students are given a discussion question during the first week of class in which they are asked to design an experiment to test the role of estrogen in altering the brain imprinting and sexual behavior of rats. Students have 1 wk to individually work outside the class to search the literature and turn in written answers for the discussion question for credit during the next week's laboratory.

The following is a typical discussion question that is provided to the students in preparation for the laboratory class.

Directions

Come to the laboratory with a typewritten answer to the question below. Present your opinions and arguments/evidence that support your opinions.

Sexual differentiation of the brain has major implications for reproduction of animals. Historically, research has demonstrated that, despite neural sex differences in the brain, lack of proper hormonal stimulation for imprinting the brain in males leads, by default, toward female sexual behaviors in the adult. Therefore, the male must activate formation of neural centers in the brain to imprint male sexual behavior. Testosterone production by the neonatal testes of males is involved with masculinization of the brain. However, testosterone is converted (aromatized) to estrogen, which is the steroid that directly stimulates the formation of neural centers involved with expressing male behavior.

You are given the assignment to develop an experiment to test the hypothesis that estrogen is involved with imprinting male sexual behavior in the neonatal rat and can override the default sex in females.

Questions. Answer the following questions to help you understand the background to the laboratory exercise to develop a well-designed experiment to determine whether estrogen is involved with imprinting male sexual behavior in neonatal rats:

- 1a. If estrogen can imprint male behavior in neonatal rats, why would endogenous estrogen production by the dam or female neonate not imprint a male brain?
- 1b. What is the mechanism of action by which testosterone induces male sexual behavior?
- 1c. Can exogenous testosterone induce imprinting of a male sexual behavior in female neonates?
- 2a. When is the neonatal rat brain sensitive to imprinting sexual behavior?
- 2b. When would you start your treatments?
- 2c. How would you remove or prevent the effect of estrogen on imprinting male sexual behavior in neonatal male rats? What could you give to block estrogen synthesis or its effects (receptors) in the brain?
- 2d. What hormone would you need to treat neonatal female rats to induce male sexual behavior?
- 2e. What would be your treatments?
- 2f. For how long would you give your treatments?
- 2g. Why must you give the same treatment to all of the neonates, regardless of sex?
- 3a. When would you make comparisons between your treatment groups?
- 3b. What biological end points would you evaluate to determine the effect of your treatments on alteration of brain sex?
- 3c. What would you measure to compare brain development and effects on reproduction in your experiment?

Equipment and Supplies

Students can be involved with treating the neonatal pups after birth, sexing and weaning pups, setting up the breeding of treatment groups, checking for the presence of vaginal “plugs,” and collecting data at the end of the experiment. All animal work related to the laboratory exercise is performed under the supervision of the instructor and is approved by the local IACUC.

The following animals and materials are necessary to conduct the experiment and collect data for student analysis:

- Instructors need an IACUC-approved animal room to house the female and male rats. Animals should be housed in conventional or isolation cages that conform with the space and stocking densities recommended in the *Guide for the Care and Use of Laboratory Animals* (see <https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf>). Standard rat chow (e.g., Laboratory Diet 5008) and water should be provided ad libitum.
- The laboratory exercise requires six pregnant female rats bred to deliver pups approximately during the second week of the course.

With four treatment groups, the two additional litters are treated with either letrozole or testosterone propionate to increase the numbers of neonates that display a disruption in mating and pregnancy. Litter size with Sprague-Dawley rats averages 13–18 pups. Commercial sources can provide rats for breeding or even time-pregnant rats (e.g., Sprague-Dawley rats can be purchased from Envigo RMS, Indianapolis, IN).

- Additional animals needed for breeding: Depending on the number of treated males and females in the experiment (Table 1), additional nontreated males (22–25) and females (60) are needed for breeding the treatment groups after the neonates reach breeding age.
- Latex gloves
- Syringes (1 ml) and needles (20 gauge)
- Scissors
- Forceps
- Scale and weigh boats
- Dissecting scope
- Microscope
- Saline solution
- Slide warmer
- Slides and coverslips
- Metric ruler
- Hormone solutions: corn oil, testosterone propionate, ICI 182,780 (ER antagonist), and letrozole (aromatase inhibitor).

Note: Latex gloves should be worn during handling, weighing, and mixing of hormone solutions.

Estrogen receptor antagonist. The stock solution of the estrogen antagonist ICI 182,780 (1-4409-25 mg; Sigma Aldrich, St. Louis, MO) is made by dissolving 25 mg in 60 μ l of DMSO, followed by the addition of 940 μ l of 100% ethanol, to produce a final volume of 1 ml (final proportions: 6% DMSO and 94% EtOH). The stock solution is stored in a refrigerator until further use. For up to 36 injections of ICI 182,780, needed to treat up to 18 neonates in a litter, we aseptically mix 144 μ l of the stock ICI 182,780 solution (25 μ g/ μ l) with 3,456 μ l of sterile corn oil in a 15-ml sterile conical tube to have 100 μ g/100 μ l for injection.

Aromatase inhibitor. The stock solution of the aromatase inhibitor letrozole (S1235-25 mg; Sellekchem, Houston, TX) is made by dissolving 25 mg in 250 μ l of methylene chloride to produce a final concentration of 100 μ g/ μ l. The stock solution is stored in a refrigerator until use. For 30–36 injections of aromatase inhibitor needed to treat 15–18 neonates in a litter, we aseptically mix 36 μ l of the stock aromatase inhibitor solution (100 μ g/ μ l) with 3,564 μ l of sterile corn oil in a 15-ml sterile conical tube to have 100 μ g/100 μ l for injection.

Testosterone propionate. The stock solution of testosterone propionate (T1875-5 g; Sigma Aldrich, St. Louis, MO) is made by dissolving 60 mg in 1 ml of 95% ethanol. The stock solution is stored at -20°C until further use. For 30–36 injections of testosterone propionate (100 μ g/100 μ l) needed to treat 15–18 neonates in a litter, we aseptically mix 60 μ l of the stock testosterone propionate solution with 3,540 μ l of sterile corn oil in a 15-ml sterile conical tube to have 100 μ g/100 μ l for injection.

Table 1. Estimate of possible animal numbers from treatment of neonates at birth

Treatment	Female	Male
Control	8	8
Testosterone propionate*	16	16
ICI 182,780	8	8
Letrozole*	16	16
Total	48	48

Values are *n*, no. of rats. *Using two litters treated at birth.

Human and Animal Subjects

Adopters of this activity are responsible for obtaining permission for animal research from their home institution. For a summary of Guiding Principles for Research Involving Animals and Human Beings, please see <https://www.physiology.org/author-info/animal-and-human-research>.

Instructions

To test the role of estrogen in imprinting sexual behavior in rats, the following experimental design and neonatal treatments are recommended after dams have pupped:

1. Experimental setup: Five to six pregnant Sprague-Dawley rats are bred to pup during the first to second week of class (15-wk semester). On the day of birth, the litter is assigned to receive one of the following treatment injections in the afternoon following birth and 48 h postbirth:

Trt 1: Control (100 μ l corn oil sc)

Trt 2: Testosterone propionate (inject 100 μ l of 100 μ g/100 μ l sc)

Trt 3: ER antagonist, ICI 182,780 (inject 100 μ l of 100 μ g/100 μ l sc)

Trt 4: Aromatase inhibitor, letrozole (inject 100 μ l of 100 μ g/100 μ l sc)

If there are two extra litters (6 female pupped) available for the experiment, testosterone propionate (Trt 2) and letrozole (Trt 4) are the best treatments to utilize for collecting additional animal data (Table 1).

The entire litter receives the same single treatment, regardless of sex, as they are somewhat difficult to sex at this time, and grooming/cleaning of pups by the dam following injections is expected to transfer testosterone and estrogen inhibitors into the milk affecting all pups.

Safety considerations: ICI 182,780 [FASLODEX (fulvestrant)] and letrozole are used clinically for the treatment of metastatic breast cancer. AstraZeneca allows the compounds to be made available for research/teaching purposes through companies such as Tocris and Sigma Aldrich. Only the instructors, laboratory technicians, or trained teaching assistants should solubilize and dilute the compounds to create the working solutions for injection. The amounts of the ER antagonist, aromatase inhibitor and testosterone propionate, used in this protocol are small, and exposure risks to the students are exceedingly low. Routine personal protective equipment, such as laboratory coats and latex or nitrile gloves, should be worn during the handling of the neonatal rats and during the injections.

The students who administer treatments to the rats are given prior instruction on the safe handling of the neonates and the proper administration of subcutaneous injections. While treatments are being administered by the students, they are assisted and supervised at all times by the instructors and/or experienced teaching assistants.

2. The litters are weaned at 21 days and sorted by sex into cages at 28 days of age. Treatment groups of males and females are allowed to reach puberty, which is at ~56 days of age.
3. Students will evaluate eight groups of rats (4 treatment groups \times 2 sexes). After reaching 60–70 days of age, males from the treatment groups are placed in cages for breeding with nontreated females (2/cage), whereas two treated females can be placed in a cage with one nontreated male. Typical design of breeding the males and females is presented in Table 1. Adjustments to the number of animals utilized in the experiment can be made, depending on the availability of nontreated males and females for breeding in the experiment. If nontreated females are limited, then the number females with treated males can be one per cage, or the number of treated males can be reduced to five

or six. Normally, there are enough breeding males in the colony to cover the needs to evaluate treated females.

- Student's plug check females for at least 7 days following the initiation of breeding. Treatment groups are split in half, and students can be divided into groups of six to plug check each morning.
 - Males remain with females following the 7-day plug checking to give time for more females to mate during the experiment. The two groups are maintained for the collection of data from their rats at the end of the experiment.
4. Treated males and all female rats are euthanized at 17–19 days after males were placed with females.
 5. Euthanasia: Rats are euthanized through CO₂ administration in a closed chamber. This method is approved by the American Veterinary Medical Association Panel on Euthanasia. Cervical dislocation is the secondary (physical) means of ensuring euthanasia.

Laboratory Measurements

Evaluation of treated males. Students should develop data sheets to obtain data on males and females by treatment males, weight, development of the reproductive tract, and fertility.

1. Record the number of females plugged for each treatment group during the first 7-day breeding period.
2. Following euthanasia, record the weight of each male at the end of the 17- to 19-day breeding period.
3. Remove each testis, and dissect the epididymis from the testis. Be sure to remove any fat from the structures. Record the weight of the testis and epididymis.
4. Place the cauda (tail) of the epididymis in warm saline on a culture dish. Use a razor blade to mince the cauda to release the sperm into the solution. Place a drop of the solution on a warm slide, and place a coverslip over the drop to view on a microscope. Evaluate and record the appearance and mobility of the sperm.
5. Remove the vesicular and coagulating gland and record the weight.
6. Carefully open the body cavity of the euthanized females that were bred to the males.
7. Record the number that are pregnant and number of implantation sites (Fig. 3).

Evaluation of treated females. Students should develop data sheets to record data on the breeding of females in the treatment groups, body weight, and fertility.

1. Record the number of females plugged for each treatment group during the first 7-day breeding period.
2. Following euthanasia, record the weight each female.
3. Carefully open the body cavity of the euthanized females and record the number that are pregnant and the number of implantation sites.

Tasks and Questions

Students record the following data on the rats, which can be placed in a spreadsheet to be shared with the entire laboratory for analysis:

Males

Treatment group: _____

Body weight: _____

Testis weight: _____

Epididymal weight: _____

Vesicular and coagulating gland weight: _____

Appearance of sperm in cauda: _____

Number of females plugged: _____

Number of females pregnant/total: _____

Number of implantation sites/pregnant female: _____

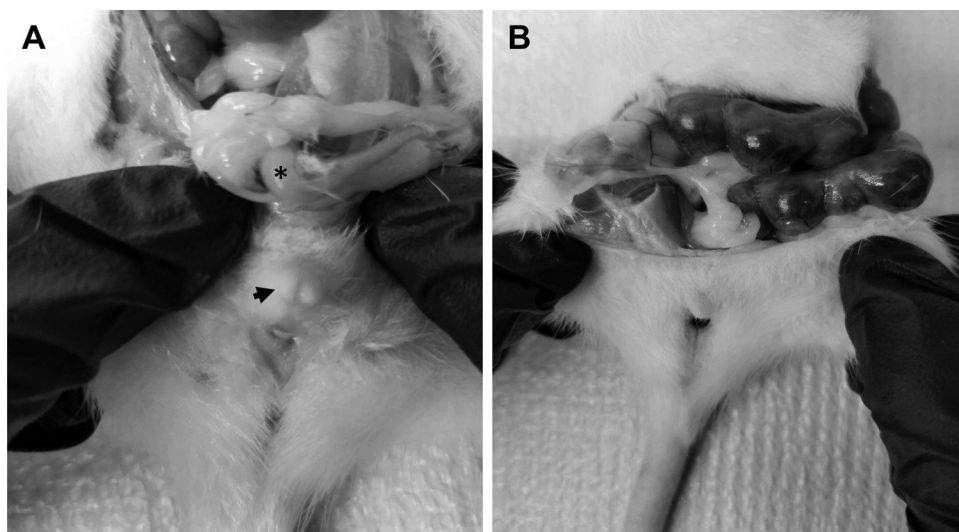


Fig. 3. Reproductive anatomy of a testosterone-treated (A) and control (B) female rat at the termination of the experiment. Note the nonpregnant uterine horn (*) of the testosterone-treated female compared with the multiple implantation sites present in the uterine horns of the control female. The external genitalia (arrowhead) is enlarged, and the vaginal opening closed in the testosterone-treated female.

Females

Treatment group: _____
 Body weight: _____
 Plugged (first 7 days of mating): _____
 Pregnant or not pregnant: _____
 Total number of implantation sites: _____

Students can be placed in groups of six to dissect the rats and record data, as well as work together to answer questions concerning the laboratory exercise.

Questions

Following the collection of data from the experiment, the instructor can provide students with the following questions to help interpret and develop conclusions from their results:

1. Did you detect any treatment differences in the number of females that were mated during the 7 days of checking for plugs in the morning?
2. Were there any treatment differences in body weight for males as well as females in the experiment? Would you expect a sex difference in body weight? Did the treatment of neonates with testosterone propionate alter the body weight of either the males or females?
3. Did any of the treatments alter development of the testis, epididymis, accessory glands (vesicular and coagulating gland) or sperm production?
4. What does the weight of the accessory glands indicate about testosterone production in the males?
5. Was the number of males mated affected by any treatment? Did ICI 182,780 (ER antagonist) or letrozole (aromatase inhibitor) affect mating behavior of the males? How would the difference in the chemical structure of ICI 182,780 compared with letrozole lead to only letrozole being capable of crossing the blood-brain barrier? Why would both not be effective at altering male sexual behavior in the neonates?
6. Was female behavior or breeding affected by any of the treatments? Would you expect either of the ICI 182,780 or letrozole treatments to alter female behavior?
7. Did testosterone propionate affect female breeding behavior, number of females that were pregnant, or implantation number?
8. Explain how treatment of neonatal rats with either testosterone propionate, ICI 182,780, or letrozole can alter imprinting brain sexual behavior.
9. Does this experiment indicate concerns for the role that environmental hormonal mimics, which includes pollution, can have on sexual brain development and imprinting?

RESULTS

Suggested Preexperiment Discussion Question and Answers

Develop an experiment to test the hypothesis that estrogen is involved with imprinting male sexual behavior in the neonatal rat and can override the default sex in females. Design an experiment to demonstrate delayed implantation in rats:

- 1a. If estrogen can imprint male behavior in neonatal rats, why would endogenous estrogen production by the dam or female neonate not imprint a male brain?
 (Answer 1a: The liver of the neonate produces α -fetoprotein, which binds to estrogens but not androgens. The binding affinity of α -fetoprotein for estrogen is greater than for the genomic ER. Therefore, estrogen cannot enter into the brain to imprint male behavior in the female neonates.)
- 1b. How is testosterone involved in development of male sexual behavior in males?
 (Answer 1b: Testosterone does not bind to α -fetoprotein, which allows diffusion across the blood-brain barrier. Neurons within regions of the brain contain aromatase, which converts testosterone to estrogens that interact with ERs to imprint the neural networks for male sexual behavior and hormone secretion.)
- 1c. Can exogenous testosterone induce imprinting of a male brain in female neonates?
 (Answer 1c: Yes. Just like in the male, testosterone will cross the blood-brain barrier and be converted to estrogens to imprint male behavior in the female.)
- 2a. When is the neonatal rat brain sensitive to imprinting sexual behavior?
 (Answer 2a: The neonatal rat brain is sensitive to imprinting right after birth to *day 5* postnatally.)
- 2b. When would you start your treatments?
 (Answer 2b: As quickly as possible following birth. Brain imprinting in the rat is usually completed by the end of *day 5* postnatally. It would be best to treat by *day 1* and again 48 h later to ensure the treatments will be effective.)
- 2c. How would you remove or prevent the effects of estrogen on imprinting male sexual behavior in neonatal

Table 2. Averages of body, testis, epididymal, accessory gland weight, and pregnancy rate of male rats at 62 days of age

Treatment	<i>n</i>	Body Weight, g	Testis Weight, g	Epididymal Weight, g	Accessory Gland Weight, g	Females Pregnant/Bred, <i>n</i>	Litter Size, <i>n</i>
Control	5	391	3.8	1.58	1.35	5/5	15.5
ICI 182,780	7	360	3.8	1.76	1.65	6/7	18
Letrozole	12	334	3.9	1.50	1.61	0/12	NP
Testosterone	11	404	2.6	1.23	1.36	8/11	16

n, No. of rats. NP (nonpregnant), none of the females bred to letrozole-treated males became pregnant during the experiment.

male rats? What could you give to block estrogen synthesis or its effects (receptors) in the brain?

(Answer 2c: The classical method would be to castrate the male neonates shortly after birth to remove the effects of testis secretion of testosterone. Without testosterone to imprint the brain, lack of ER stimulation will cause the brain to default to female behavior. It would be possible to treat the male neonates with an aromatase inhibitor to prevent conversion of testosterone to estrogen in the brain. In theory, an ER antagonist could be utilized to inhibit receptor activation in the brain.)

2d. What hormone would you need to treat neonatal female rats to induce male sexual behavior?

[Answer 2d: As demonstrated in previous studies, treatment of female neonates with testosterone propionate imprints male behavior in females. (Important to note that unconjugated testosterone is not very effective, most likely due to half-life in blood.)]

2e. What would your treatments be?

[Answer 2e: Hopefully, the class will conclude to have a control, ER antagonist, aromatase inhibitor, and testosterone treatment groups. (Important to note that any ER antagonist that has a similar structure to estradiol will bind to α -fetoprotein and fail to pass through the blood-brain barrier. The aromatase inhibitor should be utilized if selecting only one possible effective treatment to alter male behavior in the neonates.)]

2f. Why must you give the same treatment to all the neonates, regardless of sex?

[Answer 2f: First, it will be difficult to sex the neonates at this stage of development. Second, the dam will most likely lick and clean the neonates after the injections, which will be absorbed and passed to all of the littermates through the milk. Thus all neonates receive the same treatment. Since the entire litter (male and female) received the same treatment, students can discuss if treatments to change either male or female brain imprinting affected behavior of the opposite sex.]

3a. When would you make comparisons between your treatment groups?

(Answer 3a: The treated rats need to reach puberty so that breeding data can be collected. Sprague-Dawley rats are mature enough to breed usually by day 56 of age.)

3b. What would you evaluate to determine the effect of your treatments on alteration of brain sex?

[Answer 3b: Students can have multiple answers for determining the effects of the treatments. The rats mating behavior could be monitored, but would require being capable of videotaping and evaluating mating behavior for each cage during the breeding period. Blood samples could be collected to evaluate hormonal patterns of the males and females. The brains could be harvested and

stained to determine how the neural brain centers for behavior had been altered. However, these are not practical and are expensive for an undergraduate class laboratory exercise. Simply mating the rats will allow students to determine whether the rats breed (plug checking) and whether pregnancy occurs in treatment groups. Lack of plugs in the females will indicate if males are mating or females are allowing breeding in the treatment groups. Body weight will indicate if treatments alter growth of males and females. Measurement of the testis, epididymis, and accessory gland weights will indirectly indicate if testosterone is present in males compared with controls. Students can also determine if males are making sperm by evaluating the sperm in the cauda epididymis.]

Evaluation of Student Work

Students collect the data from treatment groups and combine those results with the other student groups in the laboratory. Data should be recorded in an Excel sheet and used to calculate the means for each treatment (Tables 2 and 3). If students have a background in statistics, the data can be statistically analyzed to help with interpretation of the results. The results can be developed into a laboratory report to be turned in by the students, or students can work together to make a presentation of the results in class for an open discussion led by the instructor to help ensure students understood all of the concepts for the goals and learning objectives in the laboratory exercise.

Laboratory Questions

The instructor should conduct a discussion with students following the completion of the laboratory exercise to provide an opportunity for students to express their opinions and conclusions from the experiment.

1. Did you detect any treatment differences in the number of females that were mated during the 7 days of checking for plugs in the morning?

(Students should have observed a difference in mating activity between the two treatment groups, which depend on the sex of the neonate. There should be no difference

Table 3. Body weight, pregnancy rate, and litter size of female rats at 62 days of age

Treatment	<i>n</i>	Body Weight, g	Females Pregnant/Bred, <i>n</i>	Litter Size, <i>n</i>
Control	8	269	8/8	13.5
ICI 182,780	5	294	5/5	15.6
Letrozole	8	286	6/8	14
Testosterone	12	267	0/12	NP

n, No. of rats. NP (nonpregnant), none of the testosterone-treated females bred to normal males became pregnant during the experiment.

for males plugging females in control and testosterone- and ICI 182,780-treated groups. However, letrozole-treated males rarely are able to successfully breed females. Students may observe that a few testosterone-treated females may have vaginal plugs on multiple days due to multiple rounds of successive estrous behavior in these animals. Consequently, it is important that students continue plug checks, even in females that have already “plugged.”)

2. Were there any treatment differences in body weight for males and females in the experiment? Would you expect a sex difference in body weight? Did the treatment of neonates with testosterone propionate alter the body weight of either the males or females?

[There should be a body weight difference between male and females, which is expected. The treatment groups will have some variation, but overall there is not a significant difference in body weight if ages of the animals are close (Tables 2 and 3). The question asked would be if neonatal testosterone-treated females grew faster if their brain were imprinted to be male, and if males treated with letrozole would be smaller compared with controls if they were imprinted to have a female brain.]

3. Did any of the treatments alter development of the testis, epididymis, accessory glands (vesicular and coagulating gland), or sperm production in the males? [Treatments generally have minimal effects on the testis, epididymis, accessory glands (vesicular and coagulating gland), or sperm production in the males (Table 2).]

4. What does the weight of the accessory glands indicate about testosterone production in the males?

(Since there is no treatment difference in body weight of males and the weight of the accessory glands is similar, the males are making testosterone, which should drive male sexual behavior. So the lack of breeding by letrozole-treated males is not a result of lack of testosterone production from the testis.)

5. Was the number of males that mated affected by any treatment? Did ICI 182,780 (ER antagonist) or letrozole (aromatase inhibitor) affect mating behavior of the males? Why would both not be effective at altering male sexual behavior in the neonates? What is the difference in the structure of ICI 182,780 compared with letrozole?

[Treatment of neonatal male rats with ICI 182,780 did not affect mating behavior or the number of pregnant females from the males. However, none of the females caged with letrozole-treated males should be pregnant (Table 2) because they did not mate. Students should go back to the structure of letrozole and ICI 182,780 (Fig. 2). ICI 182,780 is estradiol with an attach tail, which interferes with receptor activation. Because it has a core structure that is derived from estradiol, it will bind to α -fetoprotein and thus cannot pass through the blood-brain barrier. Therefore, ICI 182,780 does not alter male behavior, as testosterone is converted to estrogen in the brain by aromatase, and there is no receptor antagonist in the brain to interfere with male imprinting. Letrozole does not resemble estradiol and can easily pass through the blood-brain barrier to block aromatase activity, so no estrogen is made in the male brain. Therefore, in the

absence of brain estrogen synthesis, males will default to imprinting the female brain.]

6. Was female behavior or breeding affected by any of the treatments? Would you expect either of the ICI 182,780 or letrozole treatments to alter female behavior?

(Testosterone should have passed the blood-brain barrier and been converted to estrogen to imprint male brain, which affects breeding and the ability to get pregnant (Table 3). Since the female is the default sex, the lack of estrogen or binding to its receptor would not affect female behavior.)

7. Did testosterone propionate affect female breeding behavior, number of females that were pregnant, or implantation number?

[A few females may have been plugged on multiple days. Treatment of neonatal females with testosterone will remove the LH surge center from the brain, which is essential for inducing ovulation. Females may have cystic ovaries; the elevated estrogen will cause them to express estrous over numerous days, so they may mate with males for several days. However, the females do not become pregnant, despite mating (Table 3). There can be effects on the external reproductive anatomy. For example, the vagina can be closed, and an enlarged swelling of the urinary papilla can sometimes be observed (Fig. 3).]

8. Explain how treatment of neonatal rats with either testosterone propionate, ICI 182,780, or letrozole can alter imprinting brain sexual behavior.

(Students should now be able to go back to Fig. 1 and understand how estrogen is involved with imprinting the male neural network involve with expressing male sexual behavior.)

9. Does this experiment indicate concerns for the role that environmental hormonal mimics, which includes pollution, can have on sexual brain development and imprinting?

(There are a considerable number of hormonal mimics that can pass through the blood-brain barrier and bind to the ER. The hormonal mimics do not have to be identical to estrogen, but have binding sites that interact with the ERs in the brain. They will not bind to α -fetoprotein and thus can alter behavior during sensitive periods of imprinting during development.)

Inquiry Applications

This session is a combination of facilitated and guided inquiry. Following the students working through and answering the discussion question concerning designing the experiment, students lead their discussion among their group to design the experiment. The instructor follows with a discussion with all of the all students in a laboratory session to direct the design and treatments for the experiment and determination of what measurements should be made to collect data for the experiment.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.D.G., M.F.S., and J.A.G. conceived and designed research; R.D.G. and A.L.S. performed experiments; R.D.G. interpreted results of experiments; R.D.G.

prepared figures; R.D.G. drafted manuscript; R.D.G., M.F.S., and J.A.G. edited and revised manuscript; R.D.G., A.L.S., M.F.S., and J.A.G. approved final version of manuscript.

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