Ultrasonic Courtship Vocalizations of Adult Male Mice: A Laboratory Exercise Illustrating Comparable Activation by either Estradiol or Testosterone

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The importance of testosterone (T) in maintaining full expression of male mammal reproductive behavior is well documented and widely acknowledged. However, the role of estradiol (E_2) in the regulation of male reproductive behaviors is much less widely recognized or appreciated. Indeed, T can be enzymatically converted to E_2 via aromatase. Many regions within the brain of males and females are rich in aromatase, thereby offering the possibility that many of T's actions in the brain require conversion to E_2. According to this aromatization hypothesis, full expression of male reproductive behaviors is mediated via the action of E_2 in the brain. This paper describes a laboratory exercise that illustrates this phenomenon as it relates to 70 kHz ultrasonic courtship vocalizations by male mice in response to either a female conspecific or the odor of female urine. The results indicate that while castration reduces male vocalizations to either female stimulus, subcutaneous implants of estradiol benzoate (EB) or testosterone propionate (TP) are equivalent in maintaining high levels of vocalization. I find this exercise extremely useful in illustrating to students why the characterization of hormones as either masculine or feminine is erroneous. Background information is presented that will help guide class discussions. Modifications to the laboratory exercise are described that do not require surgical castration or the use of controlled substances.

Key words: rodent; copulatory behavior; hormone replacement; dihydrotestosterone; estrogen; *androgen; pheromones*

For the past ten years, I have employed rodent ultrasonic vocalizations (USVs; i.e., vocalizations above the upper frequency limits of human hearing) as one of several behavioral measures of hormone and drug effects. In my experience this simple behavior is robust, inexpensively quantified, and of inherent interest to students. Although several different rodent species produce USV in various social/environmental contexts, the majority of published studies have examined rats and house mice. In this paper I focus on the vocalizations of adult mice, provide relevant background on factors that mediate their USV production, describe a sample laboratory exercise I use in my biopsychology course, and discuss several variations on this exercise that may be of interest to other instructors.

The first published reports of mammalian USV appeared in the late 1930s (reviewed by Sales and Pye, 1974). In these earliest reports, Howard Griffin and his collaborators demonstrated that bats produced ultrasonic pips to detect, locate, and track their insect prey. Subsequent investigations of these bat vocalizations resulted in a vast literature spanning numerous sub-disciplines of biology. Among the more intriguing findings was the discovery that to evade capture many insects also produce ultrasounds to "jam" the bat USVs (Spangler, 1988). The neural mechanisms that bats use while in flight to locate prey and navigate have also been extensively investigated. Present knowledge regarding the neural mechanisms of sound localization are largely based upon this work and similar research with owls (e.g., Konishi, 1993).

The earliest reports that rodents produce ultrasonic vocalizations began to appear in the 1950s. Noirot's pioneering work focused on calls made by infants (Sales and Pye, 1974). Anderson (1954) was first to report that the adult rats also produced ultrasonic calls. 22 kHz calls by rats were subsequently identified in both males and females when isolated, injured or stressed, when confronted by a predator, during drug withdrawal, by a defeated male during intermale aggression, and by copulating males during the post-ejaculatory interval (Barfield and Geyer, 1972, 1975; Lore et al., 1976; Blanchard et al., 1991; Van derPoel and Miczek, 1991; Brudzynski and Ociepa, 1992; DeVry et al., 1993). Adult male and female rats also emit a ~50 kHz vocalization during socio-sexual interactions with conspecifics (Thomas and Barfield, 1985; White and Barfield, 1987). Pharmacological studies have begun to examine the neurotransmitter systems that mediate the adult USV (Fernandez-Gausti, 1986; Brudzynski and Barnabi, 1996; Knutson et al., 1999; Bialy et al., 2000; Brudzynski, 2001; Wintink and Brudzynski, 2001; Beck et al., 2002; Brudzynski and Pniak, 2002;).

Adult house mice (*Mus musculus*) also produce ultrasonic vocalizations (40-70 kHz; Sales, 1972). Both adult male and female mice emit these calls, but during different social contexts. Vocalizations during heterosexual encounters are made almost exclusively by the male and appear correlated with his level of sexual arousal (Nyby 1983). Females also vocalize, but much less often and typically only during same-sex encounters (Maggio and Whitney, 1985; Moles and D'Amato, 2000; D'Amato and Moles, 2001). In contrast to rats, adult mice appear not to produce USV during any contexts other than reproduction. However, the possibility that mice may produce ultrasonic calls unique to other social and environmental circumstances has not been thoroughly explored. It may be possible that detailed spectrographic and temporal

examination of calls in different contexts may reveal distinctive features of calls that occur in specific contexts and/or during specific motivational states. The availability of a wide variety of laboratory mouse strains as well as spontaneous and engineered mutants, provides an exceptional opportunity for comparative studies of USV, the genes that mediate these vocalizations and their underlying motivational state(s).

Several types of olfactory cues from females (i.e., urine, saliva, vaginal fluids) elicit USV from sexually experienced male mice (Whitney et al., 1974; Nyby et al., 1977; Byatt et al., 1986). The vomeronasal (Jacobson's) organ and the accessory olfactory system play a particularly important role in detecting the chemosignals that elicit USV (Bean, 1982; Wysocki et al., 1982). Indeed, the involvement of the vomeronasal and accessory olfactory systems responding to several other putative mouse pheromones suggests that male USV may be elicited by one or more pheromones (Halpern, 1987). However, the pheromone(s) that elicit(s) USV from males has not been isolated or identified.

In mice, as in many mammalian species, full expression of male reproductive behavior depends upon sufficient levels of circulating androgens. Castration significantly impairs male mouse copulation and ejaculation. USVs are also similarly affected, but may be restored in castrates by testosterone (Dizinno and Whitney, 1977; Nunez et al., 1978, 1984; Bean et al., 1986).

These activating effects could be mediated directly via testosterone (T) action. Alternatively, T could also be converted to other neuroactive metabolites via two metabolic pathways. The first pathway involves the conversion of T to dihydrotestosterone (DHT) by the enzyme 5α-reductase, the second involves T being converted to estradiol (E_2) via the enzyme aromatase.

Consistent with the importance of T's conversion to E_2, many regions within the brain of males and females are rich in aromatase. Moreover, the brain of males and females possess estrogen as well as androgen receptors. Therefore, circulating T could activate male reproductive behavior in several ways. T could act directly on androgen receptors, or it could do so following conversion to DHT. Alternatively, T could activate male reproductive behaviors by activation of estrogen receptors (ER) following conversion to E_2. This aromatization hypothesis, in fact, accounts for many effects of T in a number of mammalian species.

In this paper, I describe a laboratory exercise employed in my biopsychology course that may be accomplished in three weekly three-hour laboratory periods. This exercise illustrates the important activational effects of T on masculine copulatory behavior and is useful in demonstrating that T's effects within the CNS may sometimes be dependent upon conversion to E_2. This exercise is extremely useful in illustrating why the common characterization of hormones as either masculine or feminine is erroneous. Moreover, this exercise demonstrates the type of empirical evidence that serves as the basis for the aromatization hypothesis. This hypothesis has played a significant role in guiding research into the organizational effects of gonadal hormones on the developing CNS and the resulting structural and functional CNS sex differences (McEwen, 1992). The laboratory exercise has also proven to be a useful means for providing students with their first experience employing an invasive surgical procedure with a laboratory rodent.

MATERIALS AND METHODS

Subjects

The animals employed (*N* = 48) are typically the adult (> 55 day old) F1 offspring of AKR/J male and C57BL/6J female mice (Jackson Laboratory, Bar Harbor, ME) bred in the animal colony at Washington College. Adult sexually experienced males of this hybrid cross reliably emit USV to females or to female urine. All male subjects are individually housed for at least two weeks. We employ 12.5 x 17 x 28 cm polycarbonate cages, with stainless steel wire lids, and filter barrier tops (Micro-isolator Cages; Lab Products, Maywood, NJ). Social-experience animals are adult male and female mice that are systematically introduced into each subject's home cage prior to the start of behavioral testing. The female social-experience animals are also used as stimulus animals to provide the urine used as a stimulus for USV. The social-experience animals are group housed (*N* = 4) by same sex in polycarbonate cages (12.5 x 17 x 28 cm). All animals are allowed free access to food (Purina Mouse Chow) and water, and are maintained on a 12 hour light-dark schedule (lights on at 0800 hrs).

Week 1: Social Experience Regime and Baseline USVs

To insure the subjects all reliably emit USV each is given eight daily three minute encounters with a male and with a female conspecific in the subject's home cage. The order in which the male and female social-experience mouse is placed into the subject's cage is counterbalanced across days. To insure that the males are not injured during aggressive bouts, the male social-experience animal is removed from the cage when a bite attack begins.

During the final day of social experience, the amount of USV emitted by each subject is screened to verify that the purpose of the social-experience regime has been accomplished. USVs are monitored using a Mini Bat Detector (Ultra Sound Advice: London, UK), suspended 15 cm above the cage and tuned to 70 kHz. USVs are monitored by student teams, in real-time, while the female mouse is in the subject's home cage. We do not do so, but headphones can be employed to alleviate the possibility that the transduced vocalizations influence the behavior of the mice. If you have more than one bat detector, use of headphones will also allow simultaneous testing by several groups at the same time and in close proximity within the laboratory.

USVs are quantified by counting the number of five second blocks containing USV within a three minute session; thus scores range from 0 - 36. A sample response quantification sheet is included in the online supplemental materials. Animals with vocalizations in fewer than 12 blocks are excluded from further consideration. Group assignment involves matching the groups for their baseline vocalization

response.

The methods of implant construction, implant equilibration and of surgical castration employed are similar to those described for rats by Sisk and Meek (1997).

Hormone implants are constructed a week in advance of the laboratory by the instructor and/or laboratory assistant. Testosterone propionate (TP), estradiol benzoate (EB), and cholesterol are purchase from SIGMA-Aldrich (St. Louis, MO). Subcutaneous implants containing crystalline TP or 50% EB/50% cholesterol are constructed using medical grade Silastic™ tubing (i.d. = 1.47 mm, o.d. = 1.96 mm; 508-006 Dow Corning Midland, MI). Each implant is 10 mm in length, the appropriate hormone is carefully tamped into the tubing using a small laboratory spatula, and the ends sealed with wooden dowel plugs and Silastic™ medical adhesive (Silicone Type A, Dow Corning: Midland, MI). Alternatively, the wooden plugs may be omitted and the ends of the tubing sealed with silicone adhesive alone. Blank implants can be constructed using either empty Silastic™ tubing or an implant containing cholesterol (a precursor for all steroid hormones). Extreme care must be taken to prevent exposure to the hormones. Surgical mask and gloves must be worn. We construct the implants in disposable trays to eliminate the possibility of contaminating surfaces in the laboratory.

Approximately 24 hours before surgeries, each set of implants (TP, EB, and BLANK) should be placed in separate covered beakers of warm (38.0 ºC) physiological saline. This has been shown in past studies (Smith et al., 1977) to insure a steady rate of sustained hormone release from the implant.

Week 2: Surgical Castration and Hormone Replacement

Prior to the laboratory session all students receive instruction in the proper techniques for handling, restraining, and injecting laboratory rodents. I accomplish this early in the semester during a scheduled laboratory period in which the instructor and laboratory assistant demonstrate each technique and the students practice under careful supervision.

During this week students typically work in surgical teams of two to three individuals under the close supervision of the instructor and at least one student assistant who has previously completed the laboratory exercise. The typical laboratory section is limited to 12 students (a maximum of four surgical teams). The instructor and assistant demonstrate the surgical procedure for the students first and then assist each surgical team.

Each team is responsible for castrating two adult male mice while the animals are deeply anesthetized. I have found that this can be reliably accomplished by inexperienced student teams within a three-hour laboratory period. Detailed surgical procedures are provided in the online supplemental materials. At the time of surgery, three types of hormone subcutaneous implants, TP, EB, or BLANK, are surgically placed in the dorsal nape of each subject's neck. The students are not told what type of implant they are given so that they are blind to the treatment group to which the animal is assigned.

Week 3: USV to Female Urine and to Intact Female Mice

One week following surgery, each team of students is responsible for collecting behavioral data from their two mice. The purpose is to determine the amount of USV emitted in response to urine from a female conspecific. The evening prior to these tests, groups of female mice (n = 4) are placed into stainless steel metabolism cages, and urine collected overnight. Alternatively, fresh urine may be obtained by securely restraining a female mouse over a clean glass Petri dish and gently palpating the animal's bladder (Monahan and Yamazaki, 1993).

Student teams are responsible for testing the two animals that they castrated the pervious week. Teams schedule two three minute post-surgery test sessions separated by 24 – 48 hours. During the first session, each subject is tested for its vocalization response to female urine alone. Using a 1 cc syringe and 23 ga needle, 0.1 cc of urine is placed onto a cotton-tipped applicator. The urine-soaked cotton tip of the applicator is then placed into a glass test tube, the wooden end held by the experimenter broken off and discarded. The urine-soaked cotton tip is then dumped into the subject's home cage. During the next three minutes, the amount of USV is quantified as it had been during the USV baseline session two weeks prior.

Between 24 - 48 hours following the first test session, teams schedule a second test session, but this time an intact female conspecific is placed into the subject's home cage. USVs are quantified just as before. In addition, each subject's latency to mount the female is recorded.

RESULTS

Reliable between-group differences are most probable if there are a minimum of ten animals per group. I accomplish this by pooling data from animals in each experimental group with data from animals in the respective group from previous years. The pooled data are distributed to the students and each student is responsible for analyzing the results of the laboratory exercise using both descriptive and inferential statistics to make comparisons between the three groups (TP, EB, and BLANK) during each test session. We meet as a group during a regularly scheduled class period to review the results and discuss what conclusions can be drawn. Each student is responsible for writing her/his own independent laboratory report.

The pooled results from the last six laboratory classes are summarized in Figure 1. With regard to the test with a female conspecific (Figure 1A), a 3 (group) x 2 (session) ANOVA indicated significant group differences in USV, $F(2, 42) = 7.82$, $p < 0.01$. Animals in the BLANK group produced fewer ($M \pm SE$) vocalizations (19.82 ± 2.08) than animals in either the TP (27.47 ± 1.29) or EB (29.21 ± 1.24) group. Overall, fewer vocalizations were made by animals following castration (21.00 ± 1.99) than were made during screening (28.02 ± 0.79), $F(1, 42) = 7.45$, $p < 0.01$). The significant interaction effect indicated

that these differences were primarily the result of a precipitous decrease in USV among the animals in the BLANK group after castration, $F(2, 42) = 13.63$, $p < 0.01$. comparable to that seen during their initial screening. In comparison, the castrated males receiving the TP implants produced significantly more vocalizations, ($p < 0.05$). Animals that received EB implants also emitted amounts of USV similar to that of males in the TP group. Both the TP and EB groups vocalized at levels comparable to that seen during their initial screening.

Group differences in the pattern of vocalization elicited by female urine was similar, $F(2, 42) = 6.00$, $p < 0.01$. Implants of either TP or EB were equally effective in maintaining USV to females at levels significantly greater than that seen in castrated males that received BLANK implants ($p < 0.05$, see Figure 1B).

Very few animals in any group attempted to mount the female during the three minute test session, so these results are not presented.

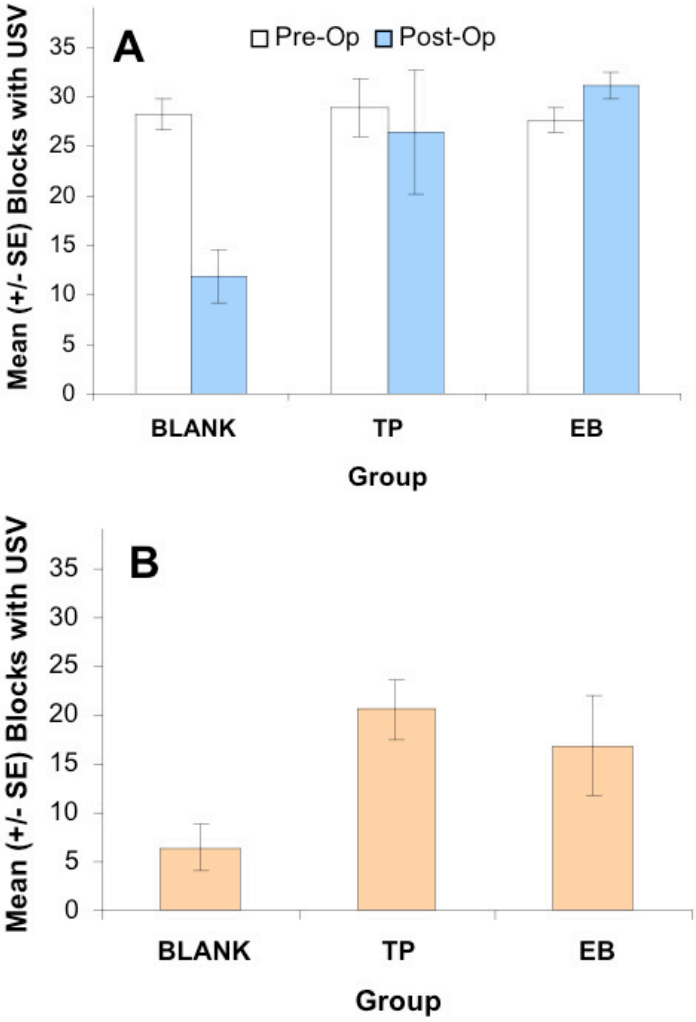

Figure 1. 70 kHz vocalizations emitted by castrated adult male mice in response to a female conspecific (A) or urine (B). Only castrated animals that received BLANK implants emitted fewer vocalizations following surgical castration than prior to castration ($p < 0.05$). Animals in the two remaining groups received implants containing estradiol benzoate (EB) or testosterone propionate (TP). Similarly, EB and TP were both effective at maintaining relatively high amounts of vocalizations in response to female urine alone, whereas animals given BLANK implants vocalized much less ($p < 0.05$).

DISCUSSION

The effectiveness of either TP or EB at maintaining castrate male USV to a female mouse or her urine is consistent with previous published findings (Nunez et al., 1978; Bean et al., 1986; Nyby et al., 1992).

Collectively these findings support the theory that T is converted to E_2 in the male CNS to regulate the expression of reproductive behaviors. Most students are not surprised that male USVs decline following castration, nor that USVs are maintained at relatively high levels in the animals given TP implants. This pattern of results is consistent with a large body of research documenting the importance of androgens, in particular testosterone, for the maintenance of male reproductive behaviors. However, the efficacy of EB in sustaining masculine behavior and the influence of the experimental context on the expression of such behavior are findings that students often find counter-intuitive. Such findings challenge students to ask more fundamental questions about what may account for these results and provide a practical example that can be used to facilitate discussions regarding the heuristic value of the aromatization hypothesis, the distinction between activational and organizational effects of hormones, and the role of gonadal hormones in the origin and maintenance of behavioral sex differences.

The Aromatization Hypothesis

According to the aromatization hypothesis, many of the effects of testosterone within the CNS may be mediated via local conversion to E_2 at target sites within the CNS. The outcome of this laboratory exercise is consistent with this hypothesis. Intra-cranial implants of T and E_2 within the medial preoptic area are also effective in restoring USV and other components of male reproductive behavior (Nyby et al., 1992). Further confirmation for the aromatization theory comes from the observation that estrogen receptor α/β knock-out ($\alpha\beta$ERKO) male mice display pronounced impairments in their reproductive behaviors including fewer USV to receptive females (Ogawa et al., 2000). Aromatase knockouts (ArKO) also show similar reproductive behavior deficits (Honda et al., 1998; Robertson et al., 2001).

In F1 C57BL/6 x AKR mice estradiol clearly plays an important role in the expression of adult male reproductive behavior. It is possible that several metabolites of T may also contribute equally or in more complex ways to influence neural processes and behavior. This may be the case for the hybrid strain in the present experiments, since concurrent treatment of castrated males with DHT and EB is more effective than EB alone (Bean et al., 1986). Such synergistic effects may be due to

the effects of DHT on peripheral androgen responsive tissues.

Strain differences may result in T or DHT, rather than E_2, playing more critical roles in mediating male reproductive behaviors in some strains. Such genetic differences might be related to differences in the distribution and activity of aromatase within the CNS. The fact that a significant number of castrated male B6D2 mice continue to copulate long after castration (Clemens et al., 1988; Wee et al., 1988; Sinchak et al., 1996) suggests that circulating levels of gonadal hormones may play a minor role in the expression of male reproductive behaviors in some strains of mice.

Of course there are bound to be some cases in which T and its non-aromatizable 5α-metabolites alone activate male reproductive behaviors. The guinea pig is an example of one such case. Alsum and Goy (1974) found that DHT, but not E_2, fully restored male copulatory behavior in this species.

However, most mammals are probably similar to the hamster, where T, DHT, and E_2 interact in some fashion to facilitate the full array of male reproductive behaviors. Like most other rodents, castration significantly impairs and androgen replacement therapy restores the copulatory behavior of male hamsters (Beach and Pauker 1949). In some instances DHT is as effective as T at restoring castrate behavior (Whalen and DeBold, 1974; Payne and Bennett, 1976), while other investigators have found DHT relatively ineffective (Christensen et al., 1973; Lisk and Benzier, 1980). Systemic treatment of castrated males with E_2 facilitates some components of male copulatory behavior, but unlike T, not all components (Tiefer, 1970). Likewise, treatment with aromatase inhibitors impairs some masculine behaviors but not others (Steel and Hutchison, 1987, 1988). Interestingly, Floody and Petropoulos (1987) found that the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) significantly reduced the amount of USV that male hamsters produce during social-sexual investigation of females. More recently, Cooper et al. (2000) found that chronic treatment (eight weeks) with the aromatase inhibitor Fadrozole™ did little to inhibit the display of reproductive behaviors in intact male Syrian hamsters. T, DHT and E_2 each appear to contribute in some way to the full expression of male hamster reproductive behavior.

The heuristic value of the aromatization hypothesis has contributed to research progress exploring hormonal mediation of reproductive behavior. The ability of E_2 to activate, and restore some components of male copulatory behavior in rodents (e.g., Pomerantz et al., 1983; Vagell and McGinnis, 1997; Wood 1996; Clancy et al., 2000) as well as other mammals (e.g., ferrets, red deer, and cynamologus monkeys; Baum, 1999), birds, lizards, and fish, illustrates that aromatization of T to E_2 may be an important mediator of male reproductive behavior in most vertebrate species.

Questions for Classroom Discussion

Is the expression of male reproductive behavior in humans dependent upon the conversion of T to E_2 in the brain?

The importance of aromatization of T in the activation of primate reproductive behavior is variable. Wallen (2001) provided a recent review of relevant primate literature in which he notes that unlike most common laboratory mammals, reproductive performance in primates is not as dependent upon hormones; that hormones affect sexual motivation rather than performance, and that performance is determined by social context as well as motivation. The findings obtained from research with human subjects are similarly equivocal (Meisel and Sachs, 1994).

The majority of relevant human studies involve observations of hypogonadal males before and after hormone replacement therapy. Hypogonadal individuals experience loss of libido and/or diminished sexual performance that can be ameliorated in a dose dependent manor by androgen replacement therapy (e.g. Davidson et al., 1979). Carani et al. (1999) compared the relative effectiveness of androgen and estrogen treatments in a rare case of an individual with an aromatase deficiency. Improvements were found in mood, heterosexual libido, frequency of sexual intercourse, masturbation, and erotic fantasies following estrogen treatment, but not following treatment with androgens. Notably, neither the individual nor his wife had complained of impairments in sexual behavior prior to treatments. Little impairment in sexual behavior was documented in another individual with aromatase deficiency (Morishima et al., 1995) or in an individual with an estrogen receptor deficiency (Smith et al., 1994). Similarly, treatment of healthy human males with either an estrogen receptor blocker or an aromatase inhibitor reportedly had no detrimental effects on their sexual behavior (Bagatell et al., 1994; Gooren, 1985). Collectively, these findings suggest that estrogen is not necessary for activation of male sexual behavior in humans, yet estrogens may act synergistically with androgens to maximally facilitate the full expression of male sexual behavior.

Is female reproductive behavior also mediated primarily by E_2?

There is ample evidence that E_2 plays a critical role in the expression of female mammal reproductive behavior (reviewed by Nelson, 2000; Wallen 2001). In many instances, full expression of female proceptive and receptive behaviors requires the combined influence of E_2 and progesterone (P). For example, both ERKO and progesterone receptor knockout (PRKO) female mice display deficits in the full expression of proceptive and receptive sexual behaviors (Lydon et al., 1995; Rissman et al., 1997).

What role if any does T play in mediating female reproductive behaviors?

The role T plays in the expression of female reproductive behaviors is unclear (see below). I require that students include a description in the discussion section of their report of an experiment of their own design that would assess the relative abilities of T, E_2 and DHT to facilitate expression of reproductive behaviors in females. We then discuss the merits of these designs when I return

the graded laboratory reports.

If T does play a role, are the effects of T dependent upon the local conversion of T to E_2 in the CNS?

The aromatization hypothesis predicts that E_2 and T might both be capable of stimulating female reproductive behavior in females, as long as the relevant target sites in the female CNS are rich in aromatase activity and that the expression of female behavior is not inhibited by effects of T mediated via androgen receptors (AR). Overall, there is very little evidence that T acts directly via AR to activate female proceptive or receptive behaviors. In contrast, there is evidence that where females are responsive to T, such activation requires T be converted to E_2 within the CNS. For example, this is the case in the female musk shrew (*Suncus murinus*; Rissman, 1991; Sharma and Rissman, 1994; Veney and Rissman, 2000).

Studies examining the role of T in the expression of female rat reproductive behavior have documented different effects on partner preference, proceptive (e.g., hopping, darting, ear-wiggling) and receptive (lordosis) components of female reproductive behavior. Acute injections of either EB or TP 48 hours prior to testing facilitated lordosis, although TP was less effective than EB. Interestingly, acute injections of the non-aromatizable androgen dihydrotestosterone propionate (DHTP) inhibited lordosis in EB-primed females (de Jonge et al., 1986a). Whereas both T and EB facilitated comparable levels of proceptive and receptive behavior in females, TP was more effective in stimulating partner preference for a male conspecific (de Jonge et al., 1986b). In contrast, Slob et al. (1987) treated ovariectomized females with either TP or EB for three weeks while measuring female preference for the proximity of either a male or female conspecific. The EB-treated females exhibited a clear preference for the male, whereas no pronounced preference for either the female or male partner among the TP-treated animals was seen. Differences in the duration of hormone treatments (acute versus chronic), and in the endocrine status of the female (intact versus ovariectomized) may account for the conflicting findings regarding partner preference. Nevertheless, both studies suggest that TP is capable of activating receptive and proceptive patterns of behavior, perhaps via aromatization to E_2. In yet another study, ovariectomized and adrenalectomized rats were treated with EB for three days followed by injections of P and/or one of three doses of TP. A synergistic influence of EB, P and TP was obtained upon the proceptive behaviors of the females. However, neither T nor P, administered independently or in combination, altered the ability of EB to stimulate lordosis (Fernandez-Guasti et al., 1991).

Collectively, these laboratory animal studies indicate that, depending upon experimental conditions, T can facilitate female reproductive behavior, although it is not clear whether this facilitation is due to direct activation of androgen receptors or the conversion to E_2 and activation of estrogen receptors. The observation that female *tfm* mice that are insensitive to androgens are nevertheless capable of breeding (Lyon and Glenister, 1980), suggests that androgens play a very modest role in activation of female reproductive behavior in mice. Examining the reproductive behavior of the female ARKO mice may help resolve this question (Yeh et al., 2002).

Perhaps the clearest evidence that endogenous T plays a role in activation of female reproductive behaviors has been obtained in studies of both human and non-human primates. For example, naturally occurring increases in levels of T are correlated with increases in sexual solicitation and receptivity in nonhuman female primates (Nelson 2000; Wallen, 2001). Similarly, peaks in T and E_2 that accompany ovulation coincide with increases in a wide range of measures used to assess sexual libido. For instance, Morris et al., (1987) reported increases in sexual intimacy among married women that corresponded with the midcycle peak in T. Of course this was a peak period for circulating E_2 as well, making it difficult to disentangle the relative contributions of the two hormones. Alexander and Sherwin (1993) found that circulating levels of free T were positively correlated with self-reported measures of sexual interest and increased responsiveness to erotic stimuli. This study as well, and others like it, suffer from the same limitation – levels of T and E_2 tend to rise and fall concurrently throughout the menstrual cycle.

To help determine if T may itself enhance sexual libido in women, investigators have turned to women receiving hormone replacement therapy following ovariectomy to halt the deleterious consequences of ovarian cancer. These studies indicate that T alone, or in addition to estrogens, increases sexual interest, arousal and activity in women (Sherwin et al., 1985; Sherwin and Gelfand 1987; Shifren et al., 2000). However, it is still unclear whether these effects are due to androgen receptor activation or if T must be converted to E_2. Moreover, methodological issues with the human studies make it difficult to obtain conclusive evidence either way (Wallen, 2001). Although supplemental T may benefit women with exceptionally low levels of T and E_2, the efficacy and safety of similar treatment for women who are experiencing sexual dysfunction but who have blood levels of T within the normal range is more controversial (Davis, 1999; Miller, 2001).

If either T or E_2 can activate male and female rodent reproductive behavior, why does such stimulation activate male reproductive behaviors in males, and female behaviors in females?

What can account for the activation of two different types of behaviors by the same hormones, in males and females? This is a very good question to ask students. The subsequent discussion commonly results in a categorical assertion that males and females are different. But this response begs several important questions, including the following one. Precisely what difference(s) account for the pattern of sex-specific behavioral responses observed? This question is a good way to introduce a discussion regarding the process of sexual differentiation and the distinction between activational and organizational roles of gonadal hormones.

Activational and Organizational Effects of Hormones

Hormones exert dramatic effects on the developing body and the central nervous system. These effects

profoundly influence the effect these same hormones have later in development. The former effects are referred to as the organizational effects of hormones, whereas the latter are the activational effects. The distinction may be applied, to the influence of gonadal hormones on development of sex differences in CNS anatomy and on the expression of behavioral sex differences during adulthood. For example the volume of the sexually dimorphic nucleus of the medial preoptic area (SDN-MPOA) in adult male rats is twice that of females (Gorski et al., 1978). This neuroanatomical dimorphism is determined during a perinatal organizational period from the 17th prenatal day through the 10th postnatal day, and possibly even as late as the 28th day of age (Rheese et al., 1990a,b; Davis et al., 1995). During this period male rats are exposed to two significant surges, pre- and post-natal, in gonadal T, whereas females are not similarly exposed (Corbier et al., 1978). Therefore it seemed likely that the SDN-MPOA was masculinized directly via the action of T on androgen receptors. However, this theory was revised after male *Tfm* rats were found to lack functioning androgen receptors, and possess feminized external genitalia, while nevertheless possessing an SDN-MPOA comparable in size to that of normal male rats (Jacobson, 1980). The SDN-MPOA in males can be feminized if the developing male rats are exposed to an estrogen receptor blocker or an aromatase inhibitor, but not an anti-androgen (Döhler et al., 1984b, 1986). These findings indicated that aromatization of T was required to masculinize the SDN-MPOA. This conclusion was confirmed by experiments examining the factors that masculinize the SDN-MPOA of females. Injecting a pregnant female with as little as a single T injection between day 18 and 20 of pregnancy was sufficient to fully masculinize the size of the SDN-MPOA of their female offspring. Interestingly, the SDN-MPOA of developing female rats can be masculinized by perinatal treatment with either T or the synthetic estrogen, diethylstilbestrol (DES; Döhler et al., 1982, 1984a; Tarttelin and Gorski, 1988). Thus it appears that the organizational effect of T on the size of the adult SDN-MPOA requires that T be aromatized to E_2.

Why doesn't the E_2 that a dam produces masculinize the brain of her female fetuses?

The apparent answer is that circulating E_2 binds to a plasma steroid binding protein, α-fetoprotein, present at elevated levels in the circulation of pregnant rats. Therefore, female fetuses are protected from the masculinizing effects that un-bound E_2 has on the developing CNS. Since α-fetoprotein does not bind DES (or T) very efficiently, DES crosses the blood-brain barrier where it masculinizes the SDN-MPOA of female fetuses. In males, T crosses into the brain where it is converted to E_2 in regions rich in aromatase such as the MPOA, causing development of the CNS to progress in a masculine pattern. So the aromatization hypothesis also nicely accounts for the organizational effects of T on the SDN-MPOA.

Sexual differentiation of the CNS mediated by hormones during the sensitive organizational period may also explain why E_2 is capable of activating male patterns of reproductive behavior in adult males and female patterns of behavior in adult females; because the brains of males and females have been "wired" differently due to the early organizational effects of T. Differences in the circuitry of the male and female brain likely accounts for the differences in the behaviors activated by E_2.

Several lines of evidence, in rodents, indicate that disrupting the early organizational effects of steroid hormones has profound effects on the sensitivity of males and females to the activational effects of hormones in adulthood. For example, prenatal stress disrupts the surge in T that occurs during the organizational period in male rats (Ward and Weisz, 1980; Weisz and Ward, 1980). Additionally, brain aromatase activity is also reduced in prenatally stressed males (Weisz et al., 1982). The result is that several sexually dimorphic structures in the CNS are incompletely masculinized (Anderson et al., 1985, 1986; Grisham et al., 1991; Kerchner and Ward, 1992). As adults, prenatally stressed males experience deficits in male patterns of reproductive behavior. Furthermore, if the stressed males are castrated in adulthood and treated with EB and P they are more likely than control males to adopt a receptive lordosis posture when mounted by a male (Ward, 1972). Conversely, female rats exposed to T prenatally have masculinized CNS structures and are more likely to show patterns of mounting and ejaculatory behavior when ovariectomized and treated with T or EB (Ward, 1984). Thus, whether the CNS has been masculinized during the organizational period determines the relative likelihood that the adult animal will display masculine or feminine reproductive behavior when hormonally stimulated in adulthood.

Alternative Laboratory Exercises

Several modified versions of this laboratory exercise may be better suited for use by instructors and students at other institutions, e.g., those without a Schedule II Drug Enforcement Agency (DEA) license. In this section I describe some of the modifications to the design that may be useful to others.

Aromatase Inhibitors, Anti-androgens, or Anti-estrogens

If it is not possible for students to perform the surgical procedures or the instructor does not possess a Schedule II DEA License, an alternative strategy utilizes s.c. injections of aromatase inhibitors (e.g., ATD, Fadrozole™), anti-androgens (e.g., flutamide) or anti-estrogens (e.g., tamoxifen). These compounds are available from Sigma-Aldrich (St Louis, MO: http://www.sigmaaldrich.com/) or Steraloids (Newport, RI: http://www.steraloids.com/). The 5α-redutase inhibitor finasteride is marketed by Merck and Co. (Whitehouse Station, NJ) as a prescription medication (Propecia™) for the treatment of male-pattern hair loss.

Time-release steroid pellets

The Silastic capsules of T and E_2 are relatively inexpensive and can be prepared in advance and stored until they are needed. However, there is a risk of steroid

exposure during capsule construction. An alternative may be to purchase time-release pellets. Innovative Research Associates (Sarasota, FL: http://www.innovrsch.com/) sells E_2 and T pellets that deliver a wide range of doses over 21, 60, or 90 days. But the cost of these implants ($435-660 for 25 21-day pellets) may be prohibitive.

Maintenance versus Restoration

In this exercise male sexual behavior (USV) was measured prior to castration, and subsequently an assessment was made of the ability of T and E_2 to maintain displays of this behavior at an equivalent level following castration. This experimental approach is called a maintenance paradigm. Alternatively, the animals could have been castrated and repeatedly tested to determine how long after castration their sexual activity would persist. Once their behavior had been determined to be significantly impaired they could then have received the hormone implants. Using this restoration paradigm, subsequent testing could be employed to determine how rapidly and effectively each hormone fully restored sexual activity to pre-castration levels.

Using Sexually Receptive Females

No attempt was made to insure sexual receptivity of the female mice used to test the USV response of male mice. In some instances, unreceptive females may aggressively repel males, thereby reducing the amount of USV that the male emits. Alternatively, the receptivity of the females could have been tested in advance using another set of intact male mice and only those females who were receptive used in the experimental test. If measures of male sexual behavior in addition to USV are to be made it is best to use such E_2 and P primed females as stimulus animals. Ovariectomized females can be brought into behavioral estrus using sequential s.c. injections of E_2 (10 mg/ml in sesame oil) and P (500 mg/ml), administered 48 hours and 4 hours prior to testing, respectively. Reasonably good results can also be obtained using E_2 and P primed intact females.

Additional Measures of Male Sexual Behaviors

In the experiments described in the present paper, very few males attempted to mount the female mice. This is most probably due to the relatively brief duration of the test session and the use of females that may not have been in behavioral estrus. In addition, other measures of male sexual behavior could be utilized. Such measures include intromission latency, "hit rate" (proportion of mount bouts accompanied by intromissions), ejaculation latency, and post-ejaculatory interval (PEI; duration of the period between the ejaculation and the next mount). To increase the opportunity that males may mount the female stimulus animal or assess effects of hormones on additional measures of sexual performance will necessitate the use of receptive female stimulus animals and longer test sessions (e.g., 30 – 45 min per subject).

Including Pre-test of USV to Female Urine

The laboratory exercise described here omitted a test of each animal's response to female urine prior to castration. Alternatively, a pre-test can be included so that there are pre- and post-treatment trials testing the relative effectiveness of a female conspecific and urine from the female to elicit USV.

Using Other Rodent Species

The laboratory exercise described here is easily adapted for rats. As noted previously, male rats produce 50 kHz pre-ejaculatory vocalizations. The 50 kHz vocalizations are activated by T, whereas DHT is relatively ineffective and E_2 even less effective (Matochik and Barfield, 1991). Production of the 22 kHz calls is not dependent upon T or T's 5α-reduced metabolites and is inhibited by estradiol dipropionate (Parrott and Barfield, 1975). Male hamsters and gerbils also produce USV during sexual interactions with female conspecifics (Sales and Pye, 1974; Nyby and Whitney, 1978). As previously discussed, the relative contributions of T, DHT, and E_2 in the hamster are complex. In the gerbil, EB and TP implants in the MPOA stimulate equivalent levels of USV and mounting in males (Holman et al., 1991).

Sources of Bat Detectors

To quantify USV, a means for detecting ultrasounds and converting them either to visible and/or audible signals is required. A relatively wide range of bat detectors, some that are relatively inexpensive, is available. A list of these is provided in the Appendix. These detectors utilize one of three methods to transform USV calls so that they are audible (i.e., heterodyne, frequency division, or time expansion). Unless the intent is to conduct detailed spectrographic Fourier analysis, an inexpensive heterodyne detector is sufficient. Broadband time-expansion detectors are sensitive across the full frequency range of the detector and preserve temporal, frequency, and intensity information needed to perform a detailed spectral analysis. Used in combination with a computer equipped with a reasonable fast processor (>300 MHz), high-speed A/D boards and appropriate sound analysis software, the time-expansion detectors allow detailed spectrographic analysis and comparisons of USV.

Several economical (< $100) detectors are suitable for this laboratory exercise and for preliminary spectrographic analysis. Shareware programs to perform spectrographic analysis of the calls are also available.

CONCLUDING SUMMARY

Although androgens, principally T and DHT, are widely recognized as important in the expression of male reproductive behaviors in mammals, the role of E_2 in these behaviors and male fertility is much less widely recognized or appreciated (Hess et al 1997; Simpson and Davies, 2000; Robertson et al., 2001). The exercise described here can provide young neuroscientists with a valuable lesson illustrating the importance of E_2 in male reproduction and help to dispel the practice of categorizing hormones as either male or female.

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Detailed laboratory handouts are available in the accompanying online supplemental materials at: www.funjournal.org/materialsKerchner.asp

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APPENDIX

Manufacturer/Retailer	Prices (USD)	Detector(s)	Address	Website & E-mail
Alana Ecology Ltd. [Peterssonn]	$40.60 – 4,631.50	H, FD, TE	The Old Primary School Church Street, Bishop's Castle Shropshire, SY9 5AE UK	www.alanaecology.com info@alanaecology.com
Bat Conservation Intern. [Peterssonn, Stag Elec.]	$89.85 - 1,070.00	H, FD, TE	Bat Conservation International P.O. Box 162603 Austin, TX 78716 USA	http://www.batcon.org batinfo@batcon.org
Convergence Technologies Inc.	$49.95 -74.95	FD	Convergence Technologies, Inc. 19 Tioga Lane, Pleasantville, NY 10570 USA	http://www.econvergence.net/
Laar Bioacoustic	$928.14 - 1,148.64	H, FD, TE	BVL von Laar Gut Klein Goernow D-19406 Klein Goernow GERMANY	http://www.laartech.biz/ info@laartech.biz
Pettersson Elektroniks AB	$196.00 – 3,977.00	H, FD, TE	Pettersson Elektronik AB Tallbacksvagen 51 S-756 45 Uppsala SWEDEN	http://www.batsound.com info@batsound.com
Stag Electronics	$67.75 - 337.75	H	Stag Electronics 120 High Street Steyning West Sussex BN44 3RD UK	www.batbox.com sales@batbox.com
Tony Messina	$40.00	FD		pw1.netcom.com/~t-rex/index.html T-Rex@ix.netcom.com
Titley Electronics	$427.00	FD	Titley Electronics PO Box 19 Ballina NSW 2478 AUSTRALIA	www.titley.com.au/index.html titley@nor.com.au
Tranquility II	$710.50	H, TE	David J Bale 3 Suffolk Street, Cheltenham GLOS GL50 2DH UK	www.users.globalnet.co.uk/~courtpan courtpan@globalnet.co.uk
Transtronics	$20.00	H	Transtronics Inc. 3209 W. 9th street Lawrence, KS 66049 USA	http://xtronics.com/kits/SK-207.htm mailto:information@xtronics.com
Ultra Sound Advice	$220.00 – 795.00	H, FD	Ultra Sound Advice 23 Aberdeen Road London N5 2UG UK	www.ultrasoundadvice.co.uk sales@ultrasoundadvice.co.uk

H = Heterodyne, FD = Frequency Division, TE = Time Expansion