Received: 30 June 2016 | Accepted: 1 September 2016

DOI 10.1002/dev.21475

RESEARCH ARTICLE

WILEY Developmental Psychobiology

Pubertal pair-housing facilitates adult sexual behavior in male rats

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Funding information
National Institutes of Health, Grant numbers: T32MH070343, MH068764

This study examined the effects of pubertal testosterone (T) and social housing manipulations on male sexual behavior in adult rats. Prepubertal rats were castrated at 21 days of age (P21) and implanted with either blank or T-releasing pellets. At the onset of puberty, P28, half the rats in each treatment group were either single- or pair-housed with a male of the same hormone condition through P56, at which time pellets were removed and all rats were single-housed. In adulthood (P84), all rats received T replacement and were tested for sexual behavior. Rats pair-housed during adolescence showed more sexual behavior and greater improvement of sexual performance over repeated tests than single-housed rats, regardless of pubertal T status. Pubertal T, however, did facilitate the frequency of anogenital investigation. Thus, in male rats, social interactions during adolescence are more important than exposure to pubertal T in enhancing adult sexual behavior.

KEYWORDS
puberty, testosterone, social housing, social isolation, male rats, anogenital investigation

1 | INTRODUCTION

Social experience facilitates rodent sexual behavior. For example, male rats raised in isolation are less likely to mate with an estrous female and take longer to initiate sexual interactions with an estrous female compared with socially raised rats (Gerall, Ward, & Gerall, 1967; Spevak, Quadagno, & Knoeppel, 1973; Thor, 1980). In addition, isolated males more frequently display aberrant or inappropriate behaviors during the mating test, for example, head shaking and leaping, compared with group-housed animals (Gerall et al., 1967). Thus, the opportunity to interact with conspecifics provided by social housing may confer the advantages of familiarity and social experience, resulting in more efficient and proficient sexual behavior.

These studies make a strong case for the importance of social experiences on sexual behavior, but they do not indicate whether social interactions during puberty might be particularly crucial for maturation of sexual behavior. The onset of puberty and the associated rise in gonadal hormone secretion occur in most rodents between 28 and 35 days of age, depending on species and sex. Puberty may be a sensitive period for social enhancement of male sexual behavior, as social housing rescues sexual behavior in rats with lesions of the medial preoptic area of the hypothalamus, but only when the lesions followed by social housing occur prepubertally, not in adulthood (Twiggs, Popolow, & Gerall, 1978). These results suggest that puberty is a period of development especially sensitive to the effects of social experiences.

In addition to social experience, pubertal testosterone (T) is necessary for optimal sexual performance and the full activation of male sexual behavior in adult rodents (Gotz & Dorner, 1976; Larsson, 1967; Schulz et al., 2004). If rats are castrated either prior to puberty or in adulthood and given testosterone propionate beginning 4 months later, those that had been castrated before puberty require more intromissions to reach ejaculation on their first sexual behavior test than do rats castrated as adults (Larsson, 1967). Another study demonstrates that mid-pubertal daily T replacement does not ameliorate the effects of prepubertal castrations in rats—these animals displayed delayed onset of mounting behavior and decreased mounting and ejaculation frequency compared to rats that were castrated mid-puberty and began T-treatment immediately following surgery (Gotz & Dorner, 1976). Together, these findings suggest that the neural circuitry regulating appropriate behavioral responses to sexual stimuli does not fully develop in the absence of testicular

hormones during early puberty. Due to some methodological confounds in these previous studies, the necessity of T during puberty for optimal adult sexual competency in male rats remains to be further clarified.

Although it is clear that both social housing and pubertal T may enhance male sexual behavior, the potential for interactions between these two variables during puberty has not been studied. In most previous social housing studies, male rats were gonadally intact (Cooke, Chowanadisai, & Breedlove, 2000; Gerall et al., 1967; Spevak et al., 1973; Twiggs et al., 1978), and therefore, it is unknown what effects pubertal housing condition might have in the absence of testicular hormones. In addition, social housing was usually initiated prior to puberty and maintained throughout the experiment, and therefore does not provide a test of whether social experience only during puberty is necessary or sufficient for enhancement of sexual behavior. The present experiments manipulated testicular hormones and social experience both separately and together during the time of puberty in order to determine the interactions between pubertal T and social housing on sexual behavior of adult male rats.

2 | METHODS

2.1 | Animals and housing

Forty nineteen-day-old male and eighty adult female (60+ days) Sprague–Dawley rats were purchased from Harlan (Madison, WI). Animals were initially single-housed in clear polycarbonate cages (44 × 22 × 21 cm) with ad libitum access to food (Telkad Rodent Diet No. 8640, Harlan) and water. The colony room was maintained on a 12:12 light-dark schedule (lights off at 1,500 hr) with ambient temperature at 21 ± 2°C. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all animal use protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

2.2 | Experimental design

One half of the males were castrated on postnatal day 21 (P21) under isoflurane gas anesthesia and ketoprofen analgesia (5 mg/kg s.c.) (See Figure 1). Half of the males were implanted subcutaneously with two testosterone (Sigma, St. Louis, MO)-filled Silastic capsules (7 mm and 15 mm length, i.d. 1.98 mm, o.d. 3.18 mm; T@P groups) at the time of castration. Pilot studies showed that these capsules deliver a T dose resulting in plasma levels of 1–2 ng/ml of testosterone. This dose is within the physiological range for adult male rats (Bodensteiner, Christianson, Siltumens, & Krzykowski, 2014; Lagunov et al., 2011; Laurenzana et al., 2002). P21 was chosen as the date of implant so that T levels would be elevated by the normal time of onset of puberty at P28. Twenty males received blank Silastic capsules (NoT@P groups) of the same dimensions at the time of castration. We did not include gonad-intact animals in the present study as we wished to standardize dose of circulating testosterone across animals, and comparisons between T@P and intact animals was not a goal. Eliminating this group also reduces the number of animals in accordance with NIH recommendations for the use of animals in research. One week following castration/capsule implantation, ten males from each hormone treatment group (T@P or NoT@P) were pair-housed with another male from the same hormone treatment group. The other ten subjects from each hormone treatment group remained single-housed. Housing and hormone treatments remained in place until the end of puberty at P56, when the capsules were removed and all animals were single-housed until the end of the experiment. At P84, all subjects were reimplanted with the same-sized subcutaneous T capsules, and sexual behavior testing began one week later. Rats were tested every 3–4 days between the ages of 91–117, for a total of six tests.

2.3 | Sexual behavior tests

Behavior testing began 1 hr into the dark phase and was conducted under dim red light. Males were allowed to acclimate to the testing chamber (60 × 31 × 30 cm glass aquarium) for 5 min. Adult stimulus female rats that were previously ovariectomized under isoflurane gas anesthesia were brought into estrus with subcutaneous injections of 10 μg estradiol benzoate and 500 μg progesterone (both purchased from Sigma) 48 hr and 4 hr before testing, respectively, as described in previous studies (Jones & Pfaus, 2014; Pfaus, Smith, & Coopersmith, 1999). Prior to the first sexual behavior test, all females were sexually inexperienced, but were screened for sexual receptivity before the onset of testing. Any female that did not display lordosis when mounted by a non-experimental stimulus male was not used in the experiment. A receptive female was then placed in the chamber and the male was allowed to interact with the female for 30 min; the testing session was videotaped for later analysis. A female was used for only one behavior test within each day of testing.

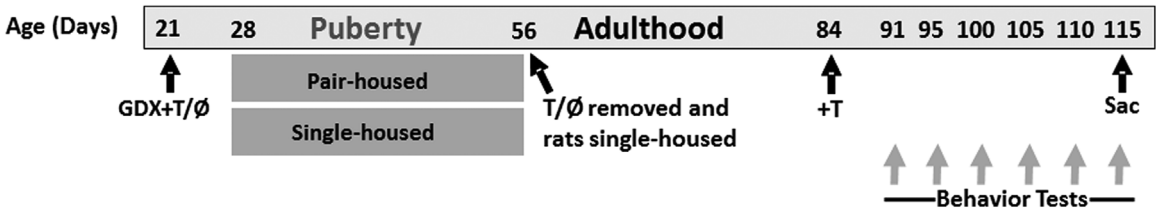

FIGURE 1 Experimental design. GDX indicates age of gonadectomy, T indicates replacement with T-containing capsules, Ø indicates blank capsules, Sac indicates age of sacrifice, and arrows indicate occurrence of sexual behavior tests

2.4 | Behavioral measurements

Sexual and non-sexual behaviors were quantified by an observer blind to the subjects' hormonal and housing conditions during puberty. Six tests were administered, as pilot studies determined that most males showed very low levels of sexual behavior on the initial test, and the literature shows that sexual performance increases with repeated testing (Drori & Folman, 1967; Larsson, 1967). Additional testing allowed us to more clearly establish differences in sexual performance between groups. Only Tests 1 and 6 were analyzed, as we expected the highest levels of behavior on the final test and the greatest differences in behavior would likely be observed between the initial and final tests.

2.4.1 | Investigatory behaviors

The frequency of and latency to sniff the female's anogenital region and other areas of the female's body by the male were quantified.

2.4.2 | Sexual Behaviors

The frequency of mounts (male grasping female's flanks and thrusting without vaginal penetration), intromissions (mount with thrusting and vaginal penetration followed by a quick dismount from the female), and ejaculations (thrusting with vaginal penetration culminating in a prolonged vigorous thrust and slow dismount from the female), as well as the latencies to display the first instance of each of these behaviors were quantified from the video recordings of the tests.

2.5 | Seminal vesicle weight and radioimmunoassay for T concentration

One hour following the end of the final behavioral test, all males received an overdose of sodium pentobarbital (150 mg/kg, i.p.). Seminal vesicles were dissected out, the fluid extracted and the dry vesicle weight was obtained. Plasma T levels were determined from blood collected via cardiac puncture at the time of sacrifice. Duplicate 50 µl samples were analyzed within a single assay using the Coat-A-Count Total T Kit (Diagnostic Products, Los Angeles, CA). In brief, calibrators were pipetted into assay tubes to serve as the standard curve for the assay. 50 µl of each unknown sample was added to a T antibody coated tube. One ml of ^{125}I total T was added to every tube, and tubes incubated in a water bath at 37 °C for 3 hr. Tubes were then decanted and placed into the gamma counter for a 1 min count. The minimum detectable concentration for the assay was .1 ng/ml. The intra-assay coefficient of variance was 7.1%; the inter-assay CV was 8.0%.

2.6 | Statistical analyses

Differences between treatment groups for seminal vesicle weights and T concentrations were analyzed using a 2-way ANOVA (T@P vs. NoT@P X single vs. pair-housed). Behavioral differences between groups were analyzed using a 2 × 2 × 2 mixed ANOVA, with the within-subject variable being test number (1 vs. 6) and the between-subject variables being pubertal T treatment and pubertal housing condition. If interactions were found between variables, 2-tailed *t* tests were used to determine differences between groups. Differences were considered significant at $p \leq .05$.

3 | RESULTS

3.1 | Investigatory behaviors

There was a main effect of both pubertal T and pubertal housing condition on the frequency of AGI of the female (Figure 2). T@P males engaged in AGI more than NoT@P males [$F(1,36) = 5.823$, $p = .021$], and those that were pair-housed engaged in AGI more than single-housed males [$F(1,36) = 5.544$, $p = .024$]. We also observed a main effect of test, as overall the four groups increased AGI frequency by Test 6 [$F(1,36) = 47.588$, $p < .001$]. Pubertal housing condition affected the latency to perform AGI, with pair-housed males displaying this behavior sooner during a test session than single-housed males [$F(1,36) = 6.628$, $p = .014$], while pubertal T treatment did not affect latencies (Table 1). All treatment groups decreased the latency to display AGI between Tests 1 and 6 [$F(1,36) = 15.226$, $p < .001$], and there was an interaction between test and pubertal housing condition by which single-housed males decreased latency more than pair-housed males [$F(1,36) = 6.596$, $p = .015$]. Follow-up *t* tests reveal this interaction resulted from pair-housed males having a shorter latency than single-housed males on Test 1 ($t(38) = -2.630$, $p = .012$), but not on Test 6 ($t(38) = .079$, $p = .937$).

3.2 | Sexual behaviors

A main effect of pubertal housing condition was found on the frequency of mounts [$F(1,36) = 6.569$, $p = .015$], intromissions [$F(1,36) = 9.857$, $p = .003$] and ejaculations [$F(1,36) = 6.400$, $p = .016$], with pair-housed males performing more of these behaviors than single-housed males (Figure 2). There were no main effects of pubertal T treatment or interactions between pubertal T and pubertal housing condition for mounts, intromissions or ejaculations. A main effect of test was observed, with all groups showing increased frequency of mounts [$F(1,36) = 16.522$, $p < .001$], intromissions [$F(1,36) = 19.151$, $p < .001$], and ejaculations[$F(1,36) = 25.600$, $p < .001$] between Tests 1 and 6 (Table 1). While no interactions between pubertal T and pubertal housing condition were observed for mounts and intromissions, there was an interaction for ejaculation frequency [$F(1,36) = 6.400$, $p = .016$; Figure 2]. However, follow-up *t* tests could not be performed to determine direction of effects because of a lack in variance of frequency scores for Test 1 (no animals performed this behavior on Test 1).

Pubertal housing condition also affected the latency to mount [$F(1,36) = 6.874$, $p = .013$], intromit [$F(1,36) = 6.741$, $p = .014$], and ejaculate [$F(1,36) = 8.540$, $p = .006$], with pair-housed males initiating mounts, intromissions and ejaculations sooner during the test than single-housed males (Table 1). Pubertal T treatment did not affect the latency to mount, intromit, or ejaculate, nor were there any interactions between treatments. There was a main effect of test on the latency to mount [$F(1,36) = 24.336$, $p < .001$], intromit [$F(1,36) = 25.670$, $p < .001$] or ejaculate [$F(1,36) = 28.132$, $p < .001$; Table 1], as there was with the

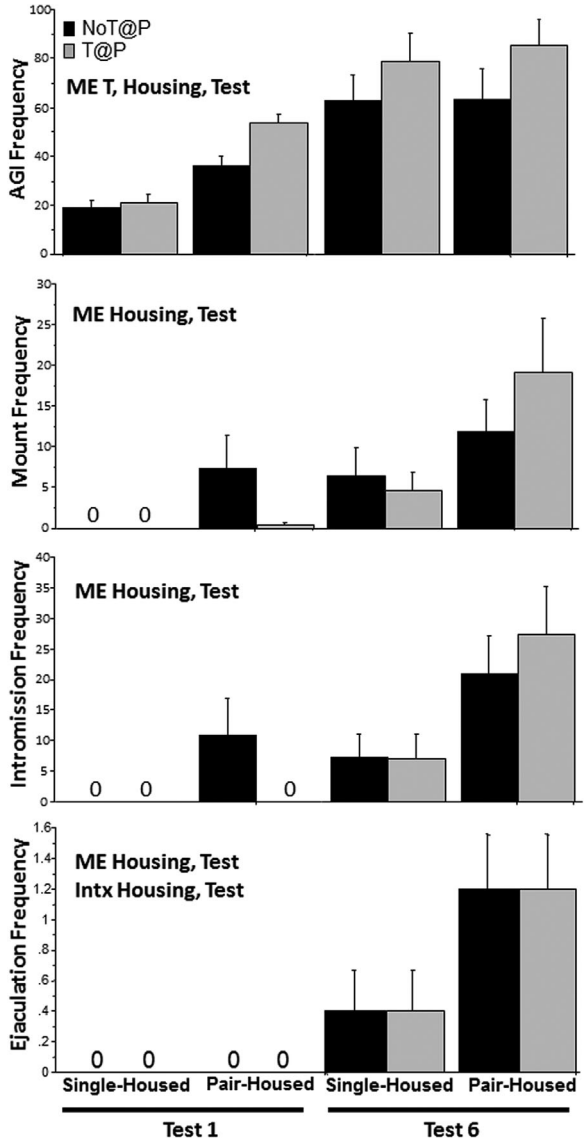

FIGURE 2 Effects of pubertal housing condition and testosterone on anogenital investigation (AGI) and sexual behaviors. Males that were pair-housed during puberty engaged in significantly more AGI than single-housed males (N = 10/group), and T@P males displayed AGI significantly more than NoT@P males. Compared to single-housed males, pair-housed males displayed a higher frequency of mounts, intromissions, and ejaculations, independent of pubertal T treatment. All groups increased the frequency of AGI, mounts, intromissions, and ejaculations over the six tests. ME, main effect, Intx interaction

frequency of these three sexual behaviors. Again, while there was no interaction between test and pubertal housing condition on latency to mount or intromit, there was an interaction on ejaculation latency [$F(1,36) = 8{,}540$, $p = .006$]. The direction of interactions between test and pubertal housing condition could not be determined due to lack of variance in latency scores for Test 1.

3.3 | Seminal vesicle weight and testosterone concentration

T@P males had greater seminal vesicle weights at the conclusion of the study than did NoT@P males [$F(1,36 = 6.309$, $p = .017$; Table 1]. There were no effects of pubertal housing condition or interactions on seminal vesicle weights. Also, there were no effects of pubertal T treatment, pubertal housing condition or interactions between treatments on plasma T levels as determined by radioimmunoassay (Table 1).

4 | DISCUSSION

These data provide evidence that pubertal housing condition is a more influential variable than pubertal T treatment on levels of adult sexual behavior in rats. Neither pubertal housing condition nor pubertal T treatment robustly affected mounts, intromissions, or ejaculations during the first test in sexually naïve males, as levels of sexual behavior were low in general upon first exposure to a receptive female. However, with repeated testing all components of sexual performance increased independently of pubertal T status, with a much more prominent increase in rats that were pair-housed during puberty (Figure 2). With respect to AGI, our data further indicate that pubertal T and pubertal housing condition interact with sexual experience in different ways. Pair-housing increased AGI independently of pubertal T status in sexually inexperienced rats (Figure 2), in agreement with results from van den Berg et al. (1999). However, by the sixth test, there was no difference in AGI between single- and pair-housed groups, and T@P animals showed the highest levels of AGI independent of pubertal housing condition. Thus, the advantage conferred by pair-housing on AGI diminishes with sexual experience, while the advantage conferred by pubertal T on AGI emerges with sexual experience.

It is possible that the socially isolated rats find social interactions in general to be anxiogenic, and this anxiety state persists despite repeated pairings with another rat. Indeed, anxiety-like behaviors in the elevated plus maze are greater in rats housed in isolation compared to group housed animals following weaning (Hellemans, Benge, & Olmstead, 2004; Kokare, Dandekar, Singru, Gupta, & Subhedar, 2010; Skelly, Chappell, Carter, & Weiner, 2015; Weiss, Pryce, Jongen-Relo, Nanz-Bahr, & Feldon, 2004). Likewise, male rats isolated for 14 days during puberty and assessed for anxiety immediately following the isolation period also showed few entries into the center of an open field chamber and a greater latency to emerge from a test start box compared to controls, indicating higher levels of anxiety (Arakawa, 2005). The effects on box emergence were not alleviated by pairing animals following the fourteen-day isolation period. Compared to group-housed rats, males isolated following weaning spend less time interacting with conspecifics in social interaction tests (Kokare et al., 2010) and emit fewer ultrasonic vocalizations while interacting with females than socially housed males (Inagaki, Kuwahara, & Tsubone, 2013a; Inagaki, Kuwahara, Tsubone, & Mori, 2013b. Isolated males also show sympathetic nervous system activation in response to female stimuli, in contrast to group-housed controls that show reproduction-associated parasympathetic activation (Inagaki et al., 2013a). These results are seemingly in contrast to the demonstration that social interactions were more rewarding to isolated adolescent rats compared to group housed rats in a conditioned place preference

TABLE 1 Behavior and physiological measures summary

		Single-housed		Pair-housed		
	Test	NoT@P	T@P	NoT@P	T@P	Effects
AGI latency	1	1.83 (.73)	1.38 (.53)	.43 (.07)	.43 (.09)	b,c,d
	6	.17 (.04)	.08 (.01)	.14 (.04)	.11 (.03)	
Mount latency	1	30.00	30.00	22.70 (3.73)	28.09 (1.92)	b,c
	6	21.25 (4.46)	23.03 (3.56)	12.26 (4.83)	12.43 (4.78)	
Intromission latency	1	30.00	30.00	23.06 (3.55)	30.00	b,c
	6	21.61 (4.29)	23.66 (3.61)	12.65 (4.73)	12.71 (4.71)	
Ejaculation latency	1	30.00	30.00	30.00	30.00	b,c,d
	6	26.25 (2.50)	26.38 (2.43)	17.37 (3.65)	17.19 (3.58)	
Seminal vesicle weight (g)		.53 (.03)	.61 (.03)	.53 (.04)	.64 (.05)	a
T concentration (ng/ml)		1.12 (.07)	1.00 (.09)	1.09 (.08)	1.09 (.08)	Ns

Data are expressed as *Mean (Standard Error of Mean)*. *N* = 10/group. AGI, anogenital investigation; T, testosterone; Ns, differences not significant. Latencies are in minutes. Latencies of 30 min indicate absence of behavior during test.
[a]Indicates main effect of pubertal T treatment.
[b]Main effect of pubertal housing condition.
[c]Main effect of test number.
[d]Interaction between pubertal housing condition and test number.

paradigm (Douglas, Varlinskaya, & Spear, 2004). However, the studies differ in isolation duration, latency to testing, and age at testing, which could explain the contradictory findings.

Previous studies in rats have found that the absence of T during adolescent development is a detriment to the performance of adult sexual behavior (Gotz & Dorner, 1976; Larsson, 1967). It was therefore surprising not to find differences in the levels of sexual behavior between males that did or did not have T during adolescence in the present studies. Methodological differences between the current and previous experiments likely account for these different outcomes. Götz and Dorner (1976) found that prepubertally castrated rats receiving T replacement (a reinstatement paradigm) at a mid-pubertal age have lower levels of behavior compared to rats castrated at mid-puberty and immediately replaced with T (maintenance paradigm). The authors suggest that, therefore, exposure to T during early puberty later on facilitates activation of sexual behavior. However, mid-pubertally castrated rats were never without T, whereas prepubertally castrated rats were without T for approximately 30 days after castration. Thus, the differences in behavior could reflect the higher efficacy of T in activating sexual behavior in a maintenance paradigm compared with a reinstatement paradigm. In addition, mid-pubertally castrated rats had already begun to display sexual behaviors before castration. It is unclear from this study what levels of previous sexual experience the mid-pubertally castrated males had, and this poses additional difficulties in interpreting the results. In another study that provided evidence for the importance of pubertal T in organizing adult male sexual behavior (Larsson, 1967), T was not replaced until 4 months following pubertal castration while in the present study T replacement for NoT@P animals occurred after only 2 months. To the best of our knowledge, no studies have investigated the effects of long-term *prepubertal* castration on sexual performance in adult rats. However, our observations suggest that prior exposure to testosterone in the T@P group did not increase later responsiveness to testosterone, as all animals displayed low levels of sexual behavior on the first behavior test, and there were no main effects of pubertal T treatment for either test (Figure 2).

The importance of social experiences during adolescence in a social species like the rat provides an interesting contrast to a solitary species like the Syrian hamster. Deprivation of T during puberty in the Syrian hamster leads to long-lasting deficits in sexual performance that cannot be ameliorated with long-term T replacement and sexual experience (Schulz et al., 2004). Male hamsters deprived of T during puberty that received non-contact exposure to estrous females throughout puberty improved sexual performance compared to males that were not exposed to females; however, their behavior never reached the levels of intact males (Schulz, Molenda-Figueira, & Sisk, 2009). This dichotomy seems evolutionarily adaptive; while the brain is organized by some factor during puberty, each species has become more sensitive to the more pertinent stimulus: conspecifics in the rat, and hormones in the hamster.

All groups showed increased sexual behaviors in Test 6 compared with Test 1. This could be due to the longer duration of T treatment over the course of the experiment. While circulating T is likely stable in all groups by the first behavior test, the neural circuitry necessary for the expression of sexual behavior can take several weeks to become receptive to the effects of hormones again (Sodersten, Damassa, & Smith, 1977). Thus, by the sixth test, the rats had been exposed to 4 weeks of testosterone treatment and circuits mediating mating behavior were more primed than in the first test. Eighteen days of testosterone treatment is sufficient to elicit sexual behavior in most rats that have been castrated and without endogenous hormones for 9 months in adulthood (Clark, Micevych, Panossian, & Keaton, 1995). However, other studies have demonstrated that, following castration four weeks earlier, adult male rats implanted with testosterone for 7 days do, in fact, mount, intromit, and ejaculate (Clark et al., 1995; McGinnis, Mirth, Zebrowski, &

Dreifuss, 1989). Alternatively, this change could be the result of sexual experience through repeated interactions with receptive females. Sexual experience facilitates sexual behavior in both gonad-intact rats (Drori & Folman, 1967), and in T-treated gonadectomized rats first tested several weeks after T replacement (Clark et al., 1995). Thus, the increase in sexual behavior could be due to long-term exposure to T, repeated sexual experience, or a combination of both factors.

It is likely that pubertal social experiences change the structure of brain regions that mediate social behaviors, and that lack of experience during critical developmental time points likely leads to the establishment of faulty connections in the brain. Interestingly, the volume and neuronal soma size in the posterodorsal medial amygdala (MePD), a sexually relevant brain nucleus, are smaller in single-housed compared to group-housed males (Cooke et al., 2000). These differences in volume and soma size were observed in parallel with the display of fewer non-contact erections and intromissions, lower hit rates and longer latency to erections in single-housed male compared to group-housed males. Social housing following weaning may improve sexual behavior by modulating the development of neural structures that comprise male mating circuit.

5 | CONCLUSION

The present study demonstrates that adolescent social experiences, such as pair-housing, are important determinants of adult sexual performance in rats. This is in contrast to earlier studies with rats and hamsters showing that T during puberty is necessary for maximum levels of adult sexual behavior (Gotz & Dorner, 1976; Larsson, 1967; Schulz et al., 2004). Testosterone exposure may serve only a permissive role, while social interactions during puberty have long-lasting facilitatory effects on mating later in life. Social deprivation during puberty may create irreversible detriments to sexual behavior through inappropriate wiring of connections between brain regions that promote social behavior, including circuits mediating sexual performance, a possibility to be explored in future studies. Because the opportunity to engage in play behaviors and to practice mating in pair-housed male rats leads to elevated sexual behavior in adulthood compared to that seen in isolates, it is therefore wise to socially house rats during adolescence when conducting behavioral experiments at any time point in development. Beyond the laboratory, captive breeding programs for social animals may also benefit from encouraging social interactions during puberty so that animals have the opportunities to practice behaviors related to sexual performance and are more effectively tuned into stimuli from potential mates.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health grants T32MH070343 (H.A.M.F.) and MH068764 (C.L.S.). The authors would like to thank Rayson Figueira and Jane Venier for their assistance with surgeries, perfusions and radioimmunoassays. We would also like to thank Chrisanthi Karanikas and Bradley Lawrence for their assistance in conducting the present studies, and Sarah Meerts, Margaret Mohr, and M. Allie Holschbach for their helpful comments on this manuscript.

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