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Peripubertal gonadal steroids are necessary for steroid-independent male sexual behavior in castrated B6D2F1 male mice

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ABSTRACT

Gonadal steroids play an integral role in male sexual behavior, and in most rodent models, this relationship is tightly coupled. However, many other species, including humans, continue to demonstrate male sex behavior in the absence of gonadal steroids, and the mechanisms that regulate steroid-independent male sex behavior are not well understood. Approximately 30% of castrated male B6D2F1 hybrid mice display male sex behavior many months after castration, allowing for the investigation of individual variation in steroidal regulation of male sex behavior. During both the perinatal and peripubertal periods of development, the organizational effects of gonadal steroids on sexual differentiation of the neural circuits controlling male sex behavior are well-documented. Several factors can alter the normal range of gonadal steroids or their receptors which may lead to the disruption of the normal processes of masculinization and defeminization. It is unknown whether the organizational effects of gonadal hormones during puberty are necessary for steroid-independent male sex behavior. However, gonadal steroids during puberty were not necessary for either testosterone or estradiol to activate male sex behavior in adulthood. Furthermore, activation of male sex behavior was initiated sooner in hybrid male mice castrated prior to puberty that were administered estradiol in adulthood compared to those that were provided testosterone. The underlying mechanisms by which gonadal hormones, during both the perinatal and peripubertal developmental periods of sexual differentiation, organize the normal maturation of neural circuitry that regulates steroid-independent male sex behavior in adult castrated B6D2F1 male mice warrants further investigation.

1. Introduction

During both the perinatal and peripubertal developmental periods, the organizational effects of gonadal steroids on sexual differentiation of the neural circuits controlling male sexual behavior are well-documented (Campbell and McGill, 1970; Luttge, 1979; Wallen and Baum, 1999). Puberty marks the end of adolescence and is an important developmental period during which, under the direction of the brain, production of testicular steroid hormones in large amounts begins. These circulating hormones mediate the transition to adulthood by binding cognate receptors in target tissues both peripherally and centrally, organizing brain circuitry (Götz and Dörner, 1976; Lenz et al., 2012). The importance of puberty in establishing adult-typical sexual behavior comes from studies of several species, in which prepubertal mating behavior is either immature or absent. For example, male Syrian hamsters do not engage in sex behavior before puberty, even after one week of dihydrotestosterone or estradiol benzoate administration (Romeo et al., 2002). Male rats will copulate prior to puberty, but only when administered testosterone (T) in amounts much greater than the threshold-dose capable of activating male sex behavior in adult castrates (Baum, 1972). However, across mammalian species there is conflicting evidence regarding the necessity of puberty for adult-typical male sex behavior (reviewed in (Schulz et al., 2004)) and little is known about the organizational role of pubertal gonadal steroids on adult-mouse reproductive behavior. From the limited evidence available, it appears that mice require perinatal but not pubertal exposure to testicular hormones for normal adult male sex behavior, as neonatal castration disrupts adult-typical male sex behavior (Quadagno et al., 1975) but prepubertal castration does not (Shrenker et al., 1985).

A substantial proportion of B6D2F1 hybrid male mice retain the full

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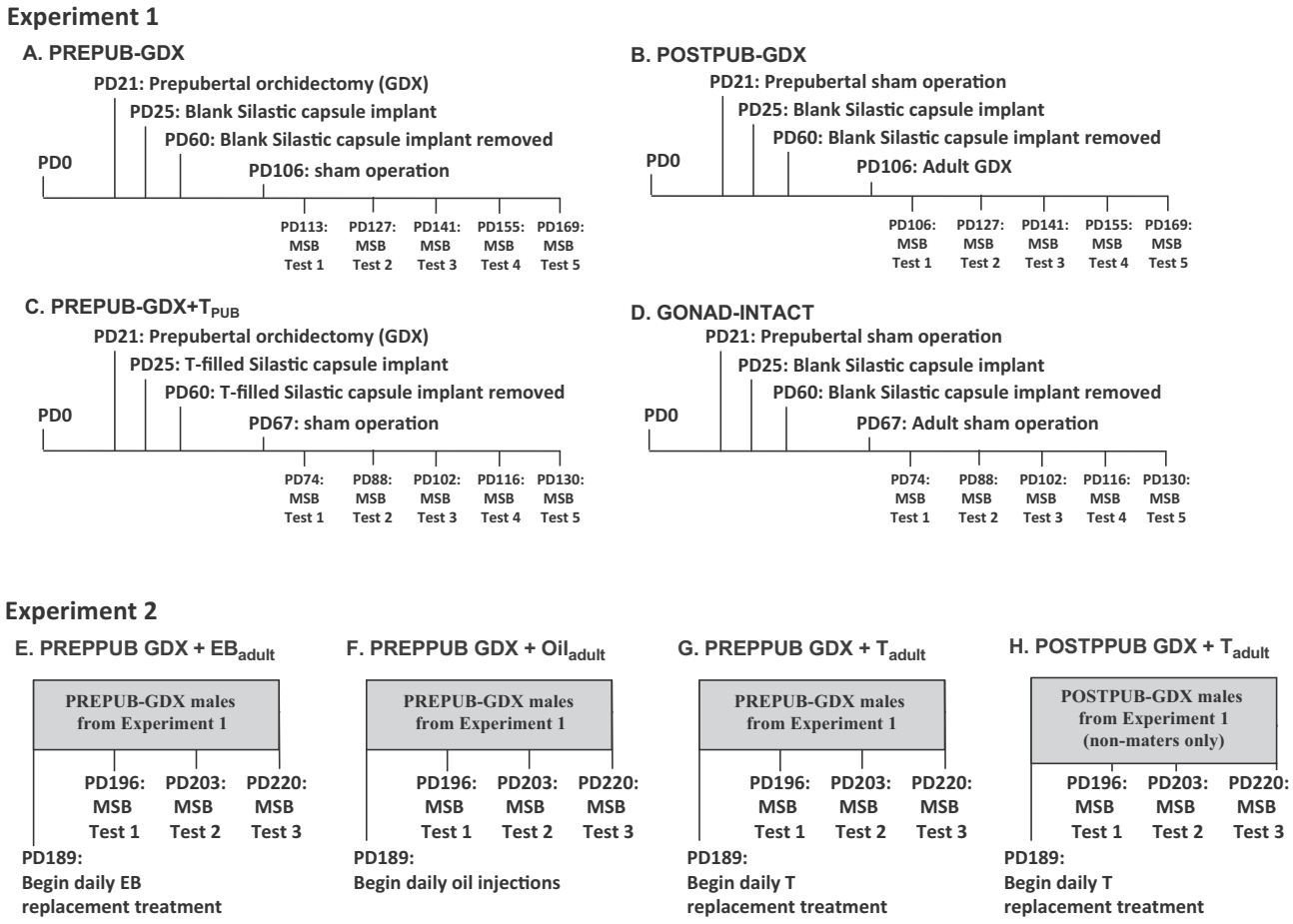

Fig. 1. Experimental timeline.

repertoire of male sex behavior after castration, up to months or even years later (herein after referred to as 'maters') (McGill and Haynes, 1973; McGill and Manning, 1976). Furthermore, the behavior persists during combined treatment with estrogen and androgen antagonists (Park et al., 2009) as well as after adrenalectomy (Thompson et al., 1976). Additionally, residual circulating T, 17 β-estradiol or dihydrotestosterone levels were not significantly different between maters and non-maters, suggesting that any residual circulating estrogens and androgens are not necessary to sustain steroid-independent male sex behavior (Sinchak and Roselli, 1996). Underlying genetic factors have been identified that regulate steroid-independent male sex behavior in adulthood, including synaptic plasticity-related genes upregulated in the maters (Bharadwaj et al., 2013; Park et al., 2010); however the developmental events required to organize the behavior remain to be uncovered. Development of reproductive behavior involves a sequence of T surges, each of which may play an important role in reaching complete maturation. To examine the impact of peripubertal steroids on the expression of adult steroid-independent male sex behavior, we castrated B6D2F1 mice on day of weaning and then assessed the expression of steroid-independent male sex behavior in adulthood. We also investigated whether replacing hormones in adulthood in B6D2F1 mice castrated prior to puberty impacted the expression of male sex behavior.

2. Material & methods

2.1. Animals

Animals were bred and raised in the animal facility at the University of Massachusetts, Boston. Male B6D2F1 hybrid mice (*Mus musculus*) were produced by crossing female C57BL/6J (B6) with male DBA/2J mice. All mice were housed in the same room, with cages individually ventilated by negative airflow to prevent inter-cage transmission of olfactory cues. The housing room was maintained on a 12:12 light cycle (lights off at 1200 h EST) and all mice were allowed ad libitum access to water and chow containing minimal phytoestrogen content (Teklad Global Rodent Diet 2016, Harlan Laboratories, Inc.). All procedures were authorized and carried out in accordance with the University of Massachusetts, Boston IACUC (#IACUC2010) & AALAC guidelines.

2.2. Assessment of male sex behavior

All experimental hybrid male mice were naïve until their first behavioral test and were individually housed once assessment of male sex behavior began. Experimental males being tested for male sex behavior were placed into Plexiglas arenas (17.8 cm w × 17.8 cm h × 25.4 cm l) with home cage bedding which had not been changed for at least 1 week, and then habituated to the arena for at least 30 min. All behavioral testing was conducted under dim red illumination in our behavior testing room, during the dark phase of the light cycle, at similar times of day.

Stimulus females for sexual behavior testing were female B6 mice (n = 23) that were ovariectomized in adulthood (postnatal day (PD) 70–74) and group-housed. Behavioral estrus was induced by priming all B6 stimulus females with subcutaneous injections of estradiol benzoate (EB, MilliporeSigma; 10 μg in 0.1 mL sesame oil) 48 h prior to testing followed by progesterone administration (P, MilliporeSigma; 400 μg in 0.05 mL sesame oil) 3–6 h prior to testing.

Behavioral testing lasted for 120 min, which began with the placement of a primed stimulus female into the arena. If the stimulus female

failed to exhibit receptivity to male mounting within the first 20 min, she was replaced with another primed female. All behavioral tests were digital video recorded and manually scored by a blinded observer for behavioral latencies including: mount latency (ML; time from the introduction of a receptive female to the first mount), intromission latency (IL; time from the introduction of a receptive female to the first intromission), and ejaculation latency (EL; interval between the first intromission and ejaculation). Counts of mounts, intromissions, and whether an ejaculation occurred were also recorded. If a stimulus female was not receptive, the scoring was reset with the introduction of the subsequent stimulus female.

2.3. Experiment 1: Are gonadal steroids, specifically T, during puberty necessary for steroid-independent male sex behavior?

In order to test whether pubertal exposure to gonadal steroids is necessary for expression of steroid-independent male sex behavior in adulthood, B6D2F1 hybrid male mice were randomly separated into several groups. In one group, 27 B6D2F1 hybrid male mice were orchidectomized on day of weaning and given a sham operation on PD 106 (PD 21; PREPUB-GDX; Fig. 1A). Given that male mice reach puberty between 5 and 7 weeks of age, when the presence of sperm in the tail of the epididymis is first detected (Martine and Claude, 1983), prepubertal orchidectomy occurred before the onset of puberty and postpubertal orchidectomy occurred well after pubertal maturation. A second group of 28 B6D2F1 hybrid male mice received a sham operation on PD 21 and were gonadectomized on PD 67 (POSTPUB-GDX; Fig. 1B). In another group, 29 male mice were gonadectomized and implanted on PD 25 with a 2-cm silastic capsule packed with crystalline T (MilliporeSigma) that was sealed with 0.5 cm of Siloxane adhesive on each end (PREPUB-GDX + T_{PUB}; Fig. 1C). This was based on the method of Wersinger et al. (1999) who employed slightly smaller silastic implants that produced physiological circulating serum T. It has been shown previously that T release from silastic capsules continues over 8 weeks (Ahmad et al., 1973), giving us confidence that our capsules were sufficient to sustain a 35 day period of T exposure between insertion on PD 25 and removal on PD 60. An additional 12 B6D2F1 hybrid male mice were given a sham operation and implanted with an empty capsule from PD 25 to PD 60 (GONAD-INTACT; Fig. 1D). After each surgery, all of the animals were isolated for 7 days to allow for recovery; all males were then group housed with littermates when possible. For post-operative analgesia, mice were given a 0.1 mg/kg injection (IP) of buprenorphine. Behavioral sex testing began on PD 106 for groups PREPUB-GDX and POSTPUB-GDX and PD 67 for groups PREPUB-GDX + T_{PUB} and GONAD-INTACT and continued for 5 bi-weekly tests (Fig. 1). All gonadectomies were performed via midline abdominal incision and cauterization under isoflurane general anesthesia. Within 24 h of the final behavioral test, GONAD-INTACT and POSTPUB-GDX males (n = 23, except a subset of 5 non-maters given T injections in Experiment 2) were rapidly decapitated under deep isoflurane anesthesia and trunk blood was collected. Remaining animals were utilized for Experiment 2.

B6D2F1 hybrid male mice were phenotyped based on their sexual performance. Hybrid males were classified as either 'maters' if the ejaculation reflex was observed on at least three out of the last four behavioral tests, including the final test, or as 'non-maters' if they did not display any components of male sex behavior during the last four tests.

2.4. Experiment 2: Is T or E2 sufficient to activate male sex behavior in males gonadectomized prior to puberty?

The animals castrated prior to puberty (from PREPUB-GDX; n = 27) and a subset of randomly chosen non-maters castrated after puberty (from POSTPUB-GDX; n = 5) from Experiment 1 were given hormone-replacement treatments 3 weeks after the last behavioral test (Fig. 1E–H). Males castrated prior to puberty were randomly chosen to receive daily s.c. injections of either vehicle (0.1 mL of sesame oil; n = 7; PREPUB-GDX + Oil_{adult}), T (20 μg/0.1 mL; n = 9; PREPUB-GDX + T_{adult}) or estradiol benzoate (EB, MilliporeSigma; 10 μg/0.1 mL; n = 11; PREPUB-GDX + EB_{adult}). The aforementioned subset of adult castrated non-maters (n = 5) received daily s.c. injections of T (20 μg/0.1 mL). These doses were chosen based on prior studies which documented reinstatement of male sex behavior in long-term gonadectomized B6D2F1 hybrid male mice (Edwards and Burge, 1971; Wee et al., 1987). Following one week of hormone-replacement treatment, 3 weekly behavioral tests for male sex behavior were conducted. On the day of testing, injections were administered to males 3–4 h prior to the introduction of the stimulus female.

Anogenital distance (AGD) of male mice from Experiment 1 (POSTPUB-GDX) and from Experiment 2 (PREPUB-GDX + T_{adult}, PREPUB-GDX + Oil_{adult}, PREPUB-GDX + EB_{adult}, and POSTPUB-GDX + T_{adult}) was measured with a fine ruler while the mice were anesthetized, just prior to sacrifice. The measurement was taken from the center of the penis to the center of the anus, to the nearest 0.5 mm. No tension was applied to the body. Within 24 h following the final behavioral test, animals were rapidly decapitated under deep isoflurane anesthesia and trunk blood was collected, centrifuged, and the serum collected and stored at −80 °C until assayed.

2.5. Testosterone ELISA assay

T was measured using a competitive Enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, catalog #ADI-900-065) according to the manufacturer's instructions. Samples from groups PREPUB-GDX + Oil_{adult}, (n = 7), PREPUB-GDX + T_{adult}, (n = 9), PREPUB-GDX + T_{PUB}, (n = 10), and GONAD-INTACT (n = 7, randomly selected) were thawed on ice and centrifuged at 1600 × *g* for 10 min at 4 °C. From the PREPUB-GDX + T_{PUB} group, randomly selected samples were chosen from males that demonstrated intromissions on the last behavioral test (n = 5) and from males that did not (n = 5). All samples were assayed in duplicate and analyzed on the same plate. A micro-plate reader was used to measure the optical density at 405 nm. The concentration of T in the samples was calculated using the 4 parameter logistic curve fitting function in PRISM (Version 6.07, Graphpad Software). As reported by the manufacturer the lower limit of detectability of the assay was 5.67 pg/mL, the intra-assay coefficient of variation was 7.8–10.8%, the inter-assay coefficient of variation is 9.3–14.6%. If the T concentration of a sample fell below the lower limit of detectability, it was assigned 5.67 pg/mL.

2.6. Statistics

To assess significance of proportions exhibiting mounting, intromission, and ejaculatory behaviors, a Generalized Estimating Equations (GEE) model with Bonferroni-corrected post hoc pairwise comparisons was used in Experiment 1 and a repeated measure ANOVA with a Tukey HSD post hoc test was used for Experiment 2. Although the proportions of animals displaying a given behavior in both experiments are represented by a binary outcome and thus well suited to a GEE model, this was not possible for Experiment 2 given the homogeneity of outcomes within groups. Therefore, outcomes were analyzed as continuous variables in the ANOVA and interpreted as qualitative. Longitudinal behavioral count and latency data (mount and intromission counts and latency to mount, intromit, or ejaculate) were analyzed using two-way repeated measures ANOVA. Familywise error rate was corrected for using the Tukey's multiple comparisons post-hoc test. Hybrid male mice that were gonadectomized prior to puberty received adult hormone treatments or oil prior to AGD measurement; distances were compared between the 3 treatment groups (T, EB and oil) using a Kruskal-Wallis test and no group differences were detected. Thus, we pooled the data of hormone and non-hormone-treated animals and

compared the AGDs of this group to those of the hybrid mice that were gonadectomized after puberty using the Mann-Whitney test. Non-parametric tests were used to assess AGD as the data were non-normal (D'Agostino-Pearson omnibus K2 normality test) and not suited for parametric testing. T concentrations were analyzed with a one-way ANOVA with a Tukey HSD post hoc test where appropriate. Two samples from the PREPUB-GDX + T_{adult} had T values greater than three standard deviations from the mean, and thus were omitted from statistical analyses. Effect sizes were estimated by calculating eta squared (η^2) for ANOVAs, phi (φ) statistics for GEE models, and the probability of superior outcome ($\hat{p}_{a_*b}$) for Kruskal-Wallis and Mann-Whitney tests. Tests were performed in Prism (Version 6.07, GraphPad Software), SPSS (version 25) and R. Observed differences were considered significant if $P < 0.05$.

3. Results

3.1. Experiment 1: B6D2F1 hybrid males castrated prior to puberty do not express steroid-independent male sex behavior in adulthood

Gonadal steroids during the peripubertal period play a significant organizational role in the sexual differentiation of male sex behavior (Campbell and McGill, 1970; Luttge, 1979; Wallen and Baum, 1999). Comparison of the proportion of animals demonstrating male sexual behaviors in each group across five tests revealed a significant interaction of treatment x test number for mounts (Wald??^{2} = 549.698, $P < 0.001$, $\varphi = 0.618$), intromissions (Wald??^{2} = 672.993, $P < 0.001$, $\varphi = 0.684$), and ejaculation (Wald??^{2} = 80.085, $P < 0.001$, $\varphi = 0.236$). Significant main effects for each treatment and test number were found for mounts (Wald??^{2} = 302.644, $P < 0.001$, $\varphi = 0.458$ and Wald??^{2} = 205.743, $P < 0.001$, $\varphi = 0.378$, respectively), intromissions (Wald??^{2} = 312.290, $P < 0.001$, $\varphi = 0.466$ and Wald??^{2} = 672.993, $P < 0.001$, $\varphi = 0.684$, respectively), and ejaculations (Wald??^{2} = 276.941, $P < 0.001$, $\varphi = 0.439$ and Wald??^{2} = 80.085, $P < 0.001$, $\varphi = 0.236$, respectively). Specifically, there was virtually a complete absence of steroid-independent male sex behavior in B6D2F1 hybrid male mice that were gonadectomized prior to puberty (PREPUB-GDX; Fig. 2); aside from an individual on Test 1, PREPUB-GDX males failed to exhibit mounts, intromissions, or the ejaculatory reflex on subsequent behavioral tests 2–5 (Fig. 2A–C). Conversely, the proportion of males gonadectomized after puberty (POSTPUB-GDX) that exhibited mounts, intromissions, and ejaculations was significantly higher relative to those gonadectomized prior to puberty at all timepoints tested (Fig. 2A–C; $P < 0.05$). Additionally, all of the hybrid males that were gonadally-intact demonstrated all three measured components of male sex behavior in each of the last 3 tests. The proportion of gonadally-intact males which demonstrated mounts, intromissions, and the ejaculatory reflex was significantly higher than PREPUB-GDX males across all 5 tests ($P < 0.05$ for all). The proportion of gonadally-intact males which demonstrated intromissions was significantly higher than each of the other three groups on Tests 4–5 and the proportion that demonstrated the ejaculatory reflex was significantly higher than each of the other three groups on Tests 3–5 (Fig. 2A–C; $P < 0.05$). The proportion of POSTPUB-GDX males that mounted was not significantly lower relative to the gonad-intact group across all 5 Tests (Fig. 2A; $P > 0.05$).

3.2. Exposure to testosterone during puberty is necessary for expression of some components of steroid-independent male sex behavior (mounting and intromissions) but not ejaculations

Because T during puberty plays a crucial role in the organization of the neural circuitry underlying male sex behavior, we hypothesized that exposure to T would be necessary for adult expression of steroid-independent male sex behavior. A significant interaction was found between treatment group and test number for mean number of mounts ($F_{(12, 368)} = 2.18$, $P < 0.05$, $\eta^2 = 0.029$), but no significant interaction was found between the two factors for mean number of intromissions (Fig. 2D–E; $F_{(12, 368)} = 1.48$, $P > 0.05$, $\eta^2 = 0.0261$). While there was no main effect of test number for either mounts or intromissions ($F_{(4, 368)} = 1.42$, $P > 0.05$, $\eta^2 = 0.008$ and $F_{(4, 368)} = 0.47$, $P > 0.05$, $\eta^2 = 0.003$ respectively), main effects of treatment were found for mounts and intromissions ($F_{(3, 92)} = 18.30$, $P < 0.05$, $\eta^2 = 0.177$ and $F_{(3, 92)} = 16.56$, $P < 0.05$, $\eta^2 = 0.151$ respectively).

B6D2F1 hybrid males that were castrated prior to puberty and provided a Silastic T-capsule during puberty (PREPUB-GDX + T_{PUB}) exhibited significantly higher levels of mounting and intromission behavior relative to prepubertal castrates not treated with T during

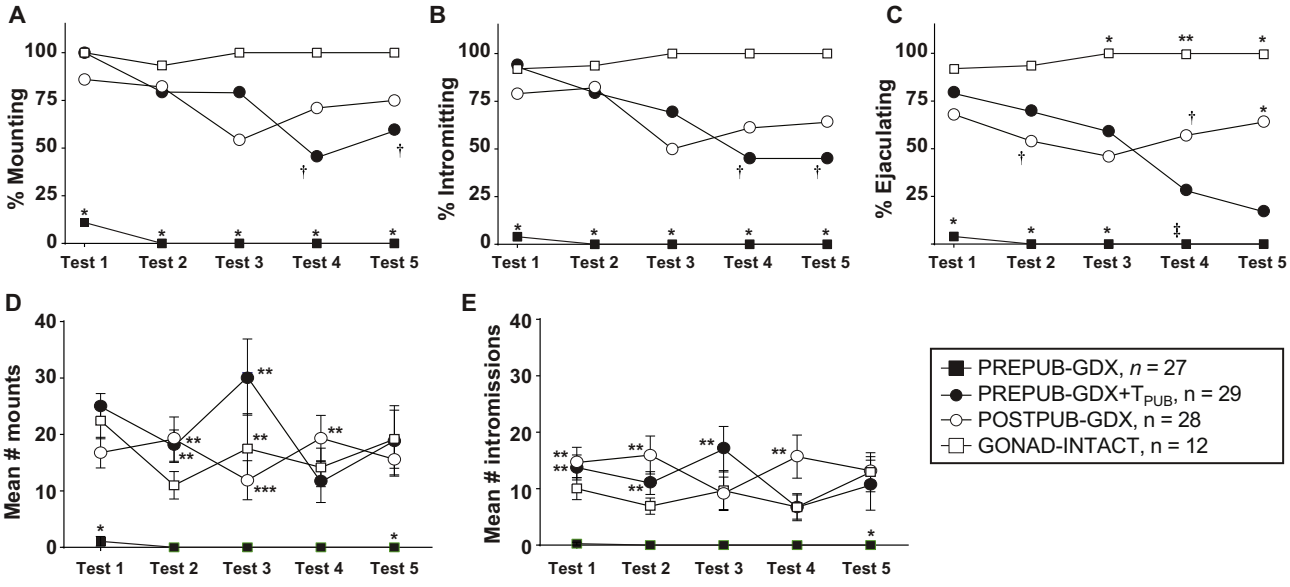

Fig. 2. Percentage of B6D2F1 hybrid male mice that displayed (A) mounting, (B) intromissions, and (C) an ejaculatory reflex. Mean + SEM number of (D) mounts and (E) intromissions. *Significantly different from the other three groups ($P < 0.05$). †Significantly different from GONAD-INTACT males ($P < 0.05$). ‡Significantly different from POSTPUB-GDX ($P < 0.05$). **Significantly different from PREPUB-GDX ($P < 0.05$). ***Significantly different from PREPUB-GDX and PREPUB-GDX + T_{PUB} ($P < 0.05$).

puberty across all five tests (Fig. 2A–B; $P < 0.05$). The proportion of PREPUB-GDX + T_{PUB} males that demonstrated the ejaculatory reflex was also significantly higher than those not exposed to T for the first three tests (Fig. 2C; $P < 0.05$), but not on the fourth nor fifth tests ($P > 0.05$). Additionally, PREPUB-GDX + T_{PUB} exhibited a similar proportion of steroid-independent male sex behavior as males gonadectomized after puberty (POSTPUB-GDX) on all behavioral tests other than Test 5, in which the relative proportion for ejaculatory behavior was significantly lower (Fig. 2; $P < 0.05$).

Relative to gonad-intact males, PREPUB-GDX + T_{PUB} demonstrated similar levels of steroid-independent male sex behavior on the first three tests ($P > 0.05$). However, a significant drop in the proportion of PREPUB-GDX + T_{PUB} males that mounted and intromitted, relative to gonad-intact males, occurred on Tests 4 and 5, as well as in the proportion that ejaculated on Tests 3–5 (Fig. 2A–C; $P < 0.05$).

Males gonadectomized after puberty demonstrated significantly more mounts and intromissions than those gonadectomized prior to puberty on all tests other than Test 3 for intromissions (Fig. 2D–E; Tukey's post-hoc tests, $P < 0.05$). The PREPUB-GDX + T_{PUB} males displayed significantly more mounts and intromissions than the PREPUB-GDX males on all tests other than Test 4 ($P < 0.05$). Relative to PREPUB-GDX males, the gonad intact males performed significantly more mounts on Tests 1, 3, and 5 as well as significantly more intromissions on Test 5 ($P < 0.05$). On Test 3, PREPUB-GDX + T_{PUB} males displayed significantly more mounts than POSTPUB-GDX males on Test 3 ($P < 0.05$).

A significant interaction of treatment x test number for mount ($F_{(12, 368)} = 5.98$, $P < 0.05$, $\eta^2 = 0.044$), intromission ($F_{(12, 368)} = 4.82$, $P < 0.05$, $\eta^2 = 0.039$), and ejaculation latencies ($F_{(12, 368)} = 5.62$, $P < 0.05$, $\eta^2 = 0.057$) was observed (Table 1); main effects for both treatment and test number were found for mount ($F_{(3, 92)} = 61.95$, $P < 0.05$, $\eta^2 = 0.478$ and $F_{(4, 368)} = 7.39$, $P < 0.05$, $\eta^2 = 0.018$, respectively), intromission ($F_{(3, 92)} = 52.39$, $P < 0.05$, $\eta^2 = 0.444$ and $F_{(4, 368)} = 4.00$, $P < 0.05$, $\eta^2 = 0.011$), and ejaculation latencies ($F_{(3, 92)} = 54.78$, $P < 0.05$ $\eta^2 = 0.396$), and $F_{(4, 368)} = 3.58$, $P < 0.05$, $\eta^2 = 0.012$). Across all 5 tests, hybrid males gonadectomized prior to puberty demonstrated significantly longer latencies to mount and intromit than the other three groups (Tukey's post-hoc tests, $P < 0.05$). Relative to that of PREPUB-GDX + T_{PUB} males, ML was significantly shorter on Test 2 for the gonad-intact group and on Tests 4 and 5 for both the POSTPUB-GDX and gonad-intact groups ($P < 0.05$). On Test 3, POSTPUB-GDX displayed a longer ML relative to gonad-intact males ($P < 0.05$). Latency to intromit for the gonad-intact group was significantly shorter than that of POSTPUB-GDX on Test 3 and PREPUB-GDX + T_{PUB} on Tests 4 and 5 ($P < 0.05$). Males gonadectomized prior to puberty demonstrated significantly longer ejaculatory latencies on Tests 1–3 for PREPUB-GDX relative to the other three groups ($P < 0.05$). Latency to ejaculate was significantly shorter for the gonad intact group relative to POSTPUB-GDX on Tests 2–5, PREPUB-GDX + T_{PUB} on Tests 3–5, and PREPUB-GDX on Tests 4 and 5 ($P < 0.05$ for all tests). On Test 4, the EL of the gonad intact group was significantly shorter than that of PREPUB-GDX ($P < 0.05$).

3.3. Experiment 2: estradiol and testosterone restores male sex behavior in prepubertal castrated B6D2F1 hybrid males

The main goal of Experiment 2 was to determine whether administration of T or EB would be sufficient to activate male sex behavior in adulthood in B6D2F1 hybrid male mice gonadectomized prior to puberty. Analyses of the proportion of animals demonstrating male sexual behaviors in each group across three tests revealed a significant interaction of treatment x test number for mounts ($F = 4.167$, $P < 0.001$, $\eta^2 = 0.068$), intromissions ($F = 5.179$, $P < 0.001$, $\eta^2 = 0.093$), and ejaculation ($F = 2.487$, $P < 0.001$, $\eta^2 = 0.104$). Significant main effects for each treatment and test number were found for mounts ($F = 80.236$, $P < 0.001$, $\eta^2 = 0.659$ and $F = 7.664$, $P < 0.001$, $\eta^2 = 0.042$, respectively), intromissions ($F = 11.362$, $P < 0.001$, $\eta^2 = 0.49$ and $F = 27.483$, $P < 0.001$, $\eta^2 = 0.165$, respectively), and ejaculations ($F = 11.925$, $P < 0.001$, $\eta^2 = 0.498$ and $F = 2.487$, $P < 0.001$, $\eta^2 = 0.104$, respectively). On Test 1 the proportion of mice, gonadectomized prior to puberty and administered daily injections of EB for one week in adulthood, that mounted, intromitted, and ejaculated was significantly higher relative to that of the prepubertal castrates that were either administered daily injections of T or vehicle (Fig. 3; $P < 0.01$).

By Test 2 (two weeks of daily hormone injections), the proportion of PREPUB-GDX + T_{adult} males that mounted and intromitted was not significantly different from those treated with EB ($P < 0.01$) and significantly higher than vehicle-treated ($P < 0.01$). However, none of the PREPUB-GDX + T_{adult} males nor the vehicle-treated males

Table 1
Mean ± SE of mount latencies (ML), intromission latencies (IL), and ejaculation latencies (EL) of B6D2F1 hybrid male mice from Experiment 1.

	Test 1		Test 2		Test 3		Test 4		Test 5	
Group	ML	SEM	ML	SEM	ML	SEM	ML	SEM	ML	SEM
POSTPUB-GDX	1632.25	458.0	1769.0	545	3584.3[c]	657.1	2396.0[b]	625.1	2099.5[b]	618.4
PREPUB-GDX	6483.8	398.1	7200.0[a]	0	7200.0[a]	0	7200.0[a]	0	7200.0[a]	0
PREPUB-GDX + T_{PUB}	477.0	46.4	1756.5	526.1	2122.6	527.4	4458.9	607.7	4031.7	580.0
GONAD-INTACT	476.8	64.0	910.1 [b]	573.5	302.8	121.5	533.2 [b]	222.3	244.3 [b]	89.5
	IL	SEM	IL	SEM	IL	SEM	IL	SEM	IL	SEM
POSTPUB-GDX	2383.9	538.3	1819.3	540.5	3984.1[c]	629.5	3204.0	656.5	2719.0	647.1
PREPUB-GDX	7044.1[a]	155.9	7200.0[a]	0	7200.0[a]	0	7200.0[a]	0	7200.0[a]	0
PREPUB-GDX + T_{PUB}	1433.4	380.2	1888.2	514.4	2924.5	578.6	4277.4[c]	604.5	4681.8[c]	579.2
GONAD-INTACT	1146.8	558.9	940.0	570.6	349	122.8	575.2	218.9	314.1	147.7
	EL	SEM	EL	SEM	EL	SEM	EL	SEM	EL	SEM
POSTPUB-GDX	3361.1	487.8	4106.5[c]	603.6	5109.6[c]	502.4	4305.7[c,d]	556.9	3762.4[a]	556.0
PREPUB-GDX	7081.5[a]	118.5	7200.0[a]	0	7200.0[a]	0	7200.0[c]	0	7200.0[c]	0
PREPUB-GDX + T_{PUB}	2875.4	425.9	3308.4	541.8	3936.0[c]	538.1	5757.0[c]	455.5	6386.1[c]	386.5
GONAD-INTACT	1655.3	525.7	1270.3	548.0	1014.6	294.6	1152.6	435.9	1050.8	353.7

[a] Significantly different than all three groups.
[b] Significantly different from PREPUB-GDX + T_{PUB}.
[c] Significantly different from GONAD-INTACT.
[d] Significantly different from PREPUB-GDX.

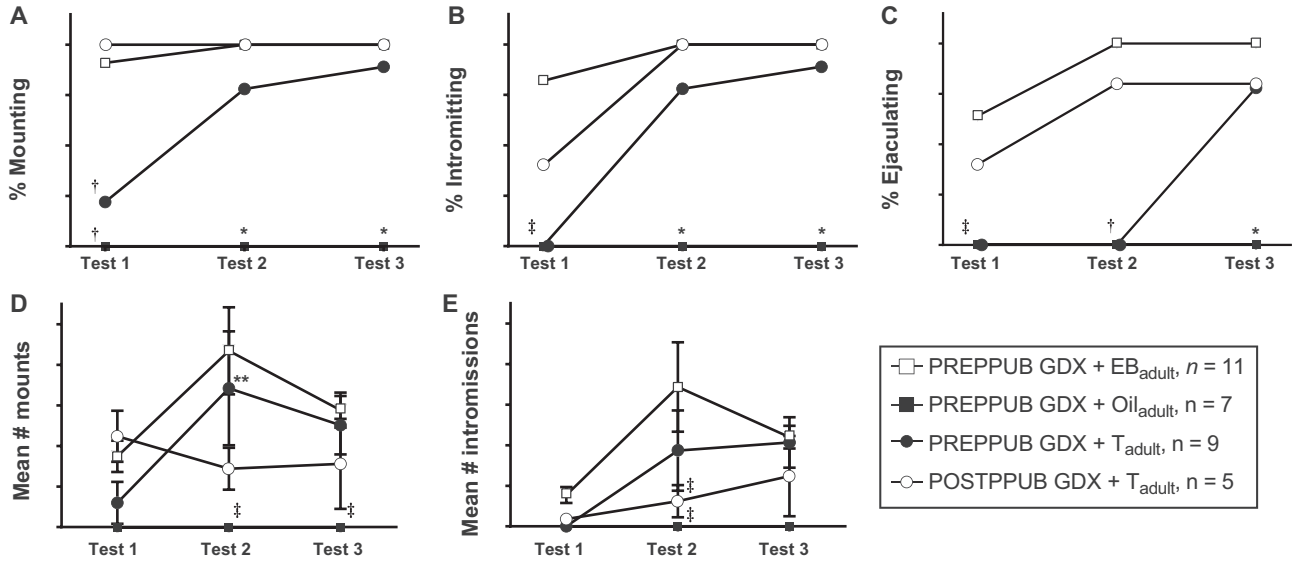

Fig. 3. Percentage of B6D2F1 hybrid male mice that displayed (A) mounting, (B) intromissions, and (C) an ejaculatory reflex. Mean + SEM number of (D) mounts and (E) intromissions. *Significantly different from the other three groups ($P < 0.05$). †Significantly different from PREPUB-GDX + EB$_{adult}$ and POSTPUB-GDX + T$_{adult}$ ($P < 0.05$). ‡Significantly different from PREPUB-GDX + EB$_{adult}$ ($P < 0.05$). **Significantly different from PREPUB-GDX + Oil$_{adult}$ ($P < 0.05$).

demonstrated ejaculation and both groups were significantly lower than EB-treated males on Test 2 ($P < 0.01$; Fig. 3).

By the end of the three-week daily hormone replacement treatment, the proportions of both T-treated and EB-treated males gonadectomized prior to puberty that displayed mounts, intromissions, and ejaculation were significantly higher than those treated with vehicle on Test 3 (Fig. 3; $P < 0.05$ for all).

3.4. Male sex behavior of adult castrated non-maters administered T relative to prepubertal castrated hybrid males injected with EB, T, or oil

Relative to the proportion of PREPUB-GDX + EB$_{adult}$ males that mounted, intromitted, or ejaculated, the proportion of POSTPUB-GDX + T$_{adult}$ males was not significantly different across all three tests (Fig. 3; $P > 0.05$). Other than Test 1 for intromissions and ejaculation, the proportion of POSTPUB-GDX + T$_{adult}$ that mounted, intromitted or ejaculated was significantly higher than that of the PREPUB-GDX + Oil$_{adult}$ for all male sex behavior tests (Fig. 3; $P < 0.05$). When compared to the PREPUB-GDX + T$_{adult}$ group, the proportion of POSTPUB-GDX + T$_{adult}$ was significantly higher on Test 1 for mounting behavior and on Test 2 for ejaculation ($P < 0.05$ for both); otherwise, there were no significant differences between the two groups for mounts, intromissions, or ejaculation on all the other tests (Fig. 3; $P > 0.05$).

A main effect of treatment for mean number of mounts and intromissions ($F_{(3, 28)} = 7.32$, $P < 0.05$, $\eta^2 = 0.204$, and $F_{(3, 28)} = 6.12$, $P < 0.05$, $\eta^2 = 0.165$ respectively) and a main effect of test number for intromissions was observed ($F_{(2, 56)} = 4.55$, $P < 0.05$, $\eta^2 = 0.0727$). However, in general, the mean number of mounts or intromissions across all three behavioral tests among the four groups did not differ. Differences between groups were only observed on Tests 2 and 3 between PREPUB-GDX + Oil$_{adult}$ and PREPUB-GDX + EB$_{adult}$ and on Test 2 between PREPUB-GDX + Oil$_{adult}$ and PREPUB-GDX + T$_{adult}$ for mean number of mounts ($P < 0.05$). Mean number of intromissions were significantly different between the PREPUB-GDX + EB$_{adult}$, PREPUB-GDX + Oil$_{adult}$ and POSTPUB-GDX + T$_{adult}$ groups on Test 2 (Fig. 3E; $P < 0.5$).

There was a significant interaction between treatment and test number for mount ($F_{(6,56)} = 5.66$, $P < 0.05$, $\eta^2 = 0.0669$), intromission ($F_{(6,56)} = 6.47$, $P < 0.05$, $\eta^2 = 0.0974$), and ejaculation latencies ($F_{(6,56)} = 3.49$, $P < 0.05$, $\eta^2 = 0.0734$). Main effects of treatment and test number were observed for mount ($F_{(3, 28)} = 49.91$, $P < 0.05$, $\eta^2 = 0.6654$, and $F_{(2, 56)} = 8.34$, $P < 0.0$, $\eta^2 = 0.03295$ respectively), intromission ($F_{(3, 28)} = 38.32$, $P < 0.05$, $\eta^2 = 0.47692$, and $F_{(2, 56)} = 33.68$, $P < 0.05$, $\eta^2 = 0.169$ respectively), and ejaculation latencies ($F_{(3, 28)} = 28.20$, $P < 0.05$, $\eta^2 = 0.4643$, and $F_{(2, 56)} = 16.12$, $P < 0.05$, $\eta^2 = 0.1128$ respectively; Table 2). Latencies to mount and intromit were significantly shorter for PREPUB-GDX + Oil$_{adult}$ on Tests 2 and 3 compared to the other three groups (Table 2; $P < 0.05$). On Test 1, PREPUB-GDX + Oil$_{adult}$ displayed a longer ML relative to PREPUB-GDX + EB$_{adult}$ and PREPUB-GDX + T$_{adult}$ ($P < 0.05$). Also on Test 1, ML for PREPUB-GDX + EB$_{adult}$ and PREPUB-GDX + T$_{adult}$ was significantly shorter relative to PREPUB-GDX + T$_{adult}$ ($P < 0.05$). Latency to intromit for PREPUB-GDX + Oil$_{adult}$ and PREPUB-GDX + T$_{adult}$ was significantly longer than that of PREPUB-GDX + EB$_{adult}$ on Test 3 ($P < 0.05$). Ejaculation latency was significantly longer on Test 3 for PREPUB-GDX + Oil$_{adult}$ relative to the other three groups (Table 2; $P < 0.05$). Latency to ejaculate was significantly shorter for PREPUB-GDX + EB$_{adult}$ relative to the PREPUB-GDX + Oil$_{adult}$ and PREPUB-GDX + T$_{adult}$ on Tests 1–2 ($P < 0.05$). On Test 2, the EL of PREPUB-GDX + T$_{adult}$ was significantly longer than that of the POSTPUB-GDX + T$_{adult}$ ($P < 0.05$).

3.4.1. Anogenital distance

There was no difference between the median AGD of the hybrid maters relative to that of the non-maters (B6D2F1 hybrid male mice from the POSTPUB-GDX group from Experiment 1; $U = 58$, $P > 0.05$, $\hat{p}_{a*b} = 0.483$). Furthermore, there were no significant AGD differences among male mice gonadectomized prior to puberty that were treated with T, EB, or oil in adulthood ($H_{(2)} = 2.22$, $P > 0.05$, $\hat{p}_{a*b} = 0.003$). Therefore, the AGD measurements of these three groups were collapsed and compared to the AGD of the adult castrates on day of sacrifice to determine the potential effect of peripubertal gonadal hormones on AGD. The median AGD of hybrid males that were gonadectomized after puberty was significantly longer than those gonadectomized before puberty (Fig. 4; $U = 135.5$, $P < 0.05$, $\hat{p}_{a*b} = 0.218$).

3.4.2. Testosterone

T concentrations of males that intromitted on the last behavioral test from PREPUB-GDX + T$_{PUB}$ (5.67 ± 0 pg/mL) and those that did not (99.29 ± 93.62 pg/mL) were not significantly different from each other (Fig. 5). T concentrations from PREPUB-GDX + Oil$_{adult}$

Table 2
Mean ± SE of mount latencies (ML), intromission latencies (IL), and ejaculation latencies (EL) of B6D2F1 hybrid male mice from Experiment 2.

	Test 1		Test 2		Test 3	
Group	ML	SEM	ML	SEM	ML	SEM
PREPUB-GDX + EB_{adult}	928.6[c]	658.0	45.6	4.5	56.5	10.5
PREPUB-GDX + Oil_{adult}	7200.0[b,d]	0.0	7200.0[a]	0.0	7200.0[a]	0.0
PREPUB-GDX + T_{adult}	5621.9	1043.8	1673.1	1044.8	856.9	792.9
POSTPUB-GDX + T_{adult}	93.6[c]	71.4	26.6	4.1	24.4	7.8
	IL	SEM	IL	SEM	IL	SEM
PREPUB-GDX + EB_{adult}	2089.9	990.1	107.5	34.6	61.5	10.1
PREPUB-GDX + Oil_{adult}	7200.0[b]	0.0	7200.0[a]	0.0	7200.0[a]	0.0
PREPUB-GDX + T_{adult}	7200.0[b]	0.0	1803.9	1021.2	876.8	790.5
POSTPUB-GDX + T_{adult}	5323.2	1362.7	1573.0	1185.0	51.2	17.7
	EL	SEM	EL	SEM	EL	SEM
PREPUB-GDX + EB_{adult}	4013.3	843.6	2215.3	401.8	1097.6	235.2
PREPUB-GDX + Oil_{adult}	7200.0[b]	0.0	7200.0[b]	0.0	7200.0[a]	0.0
PREPUB-GDX + T_{adult}	7200.0[b]	0.0	7200.0[b]	0.0	3576.0	1042.5
POSTPUB-GDX + T_{adult}	5379.2	1329.4	1935.0[c]	1336.1	1841.2	1371.3

[a] Significantly different than all three groups.
[b] Significantly different from PREPUB-GDX + EB_{adult}.
[c] Significantly different from PREPUB-GDX + T_{adult}.
[d] Significantly different from POSTPUB-GDX + T_{adult}.

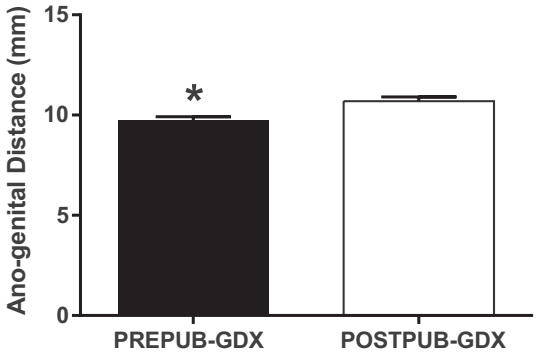

Fig. 4. Mean + SEM anogenital distance (AGD) of B6D2F1 hybrid male mice. There were no significant differences in AGD among prepubertal castrates treated with T, EB, or oil in adulthood. Therefore, the AGD measurements of these three groups were collapsed and compared to the mean AGD of the adult castrates on day of sacrifice to determine the potential effect of prepubertal gonadal hormones on AGD. *Significantly lower than POSTPUB-GDX males ($P < 0.05$).

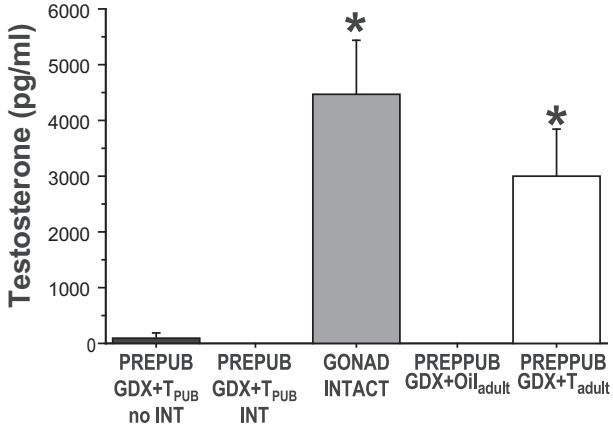

Fig. 5. Mean + SEM serum testosterone concentration. *Significantly higher than PREPUB-GDX + Oil_{adult} and PREPUB-GDX + T_{PUB} ($P < 0.05$).

(5.67 ± 0 pg/mL) and PREPUB-GDX + T_{PUB} males (both groups) were significantly lower than both the PREPUB-GDX + T_{adult} (2985.19 ± 846.11 pg/mL) and GONAD-INTACT males (4455.9 ± 990.559 pg/mL; $F_{(4,\ 26)} = 10.19$, $P < 0.05$, $\eta^2 = 0.6$; Fig. 5).

4. Discussion

The results of our study demonstrate, for the first time, that gonadal hormone exposure during puberty is necessary for adult expression of steroid-independent male sex behavior, and T treatment during puberty is sufficient to rescue the adult expression of some components of steroid-independent male sex behavior (mounting and intromitting) but not all (ejaculations) in B6D2F1 male mice gonadectomized prior to puberty (Experiment 1; Fig. 2). Expression of mounting and intromitting in males treated with T during puberty was independent of circulating T, as T concentrations in this group were minimal when measured immediately after the last test for male sex behavior (Fig. 5). Although T was not measured prior to the last test, T decreases to undetectable levels within 24 h of removal of a T-filled Silastic capsule (Krey and McGinnis, 1990). Thus, it seems unlikely that male sex behavior was activated by any remaining levels of T 14 days after removal of the capsule (Test 1, PD74) in the PREPUB-GDX + T_{PUB} group. However, because lasting activational effects of T on male sex behavior are known to extend beyond the point where circulating T has decreased, it remains possible that lasting activational effects may have been present 14 days after removal of the T-filled Silastic capsule, but highly unlikely 70 days after hormone withdrawal (Test 5, PD130).

Other than a single individual on the initial behavioral test, none of the B6D2F1 hybrid males that were gonadectomized prior to puberty displayed steroid-independent male sex behavior when tested in adulthood; conversely, a high proportion of the hybrid males that were gonadectomized after puberty ('maters') displayed steroid-independent male sex behavior as 46%–64% demonstrated the ejaculatory reflex on Tests 2–5 (Fig. 2). Our results are analogous to those discovered in a similar study conducted with male Siberian hamsters, another species in which a high proportion of males demonstrate steroid-independent male sex behavior after long-term gonadectomy; castration prior to puberty also prevented steroid-independent male sex behavior in adult male Siberian hamsters (Costantini et al., 2007). Our findings also

correspond with prior studies in male Syrian hamsters that have shown puberty is a developmental period in which testicular hormones may organize the underlying neural circuitry regulating male sex behavior (Schulz et al., 2004, 2009; Schulz and Sisk, 2006). Depriving males of T during puberty results in long-lasting deficits in steroid-dependent reproductive behavior in male Syrian hamsters, and as our results demonstrate, deprivation of peripubertal T leads to the complete elimination of steroid-independent male sex behavior in castrated B6D2F1 hybrid male mice.

While the proportion of adult castrated hybrid mice and prepubertal castrates that were treated with T during puberty did not differ between those that mounted and intromitted, there was a significant decrease in the percentage of prepubertal castrates, treated with T during puberty, that demonstrated the ejaculatory reflex on the last two tests relative to that of adult castrates (Fig. 2). These results indicate that gonadal steroids other than T may also play a role in organizing the neural underpinnings important for sustaining the ejaculatory reflex after long-term absence of gonadal steroids during puberty. Evidence from ERβ KO mice supports this idea, with ERβ KO mice expressing all components of male sex behavior significantly later and with an altered pattern of copulation than controls (Temple et al., 2003).

The current study also demonstrates that exposure to gonadal steroids during puberty is not necessary for activation of male sex behavior in the B6D2F1 hybrid male mouse, as treatment with either T or EB in adulthood was sufficient to activate male sex behavior in mice that were gonadectomized prior to puberty (Experiment 2; Fig. 3). Taken together, these results suggest that gonadal hormones during the *perinatal* critical period of sexual differentiation play a significant role in the organization of neural circuitry underlying steroid-*dependent* male sex behavior in B6D2F1 hybrid male mice, while *peripubertal* gonadal hormones are necessary for finishing the process of refining the neural organization underlying certain components of steroid-*independent* male sex behavior, specifically mounting and intromitting. Our results fit the theory that puberty, and concomitant organization of adult social behavior, is an extended window of decreasing postnatal sensitivity to steroid hormones (Arnold and Breedlove, 1985; Schulz and Sisk, 2016). Even within the perinatal critical period, further refinement of the organization of neural circuitry regulating male sex behavior may occur. In male mice, a prenatal T surge occurs on approximately day 17 of gestation followed by a second T surge on the day of birth (PD 0) (Burns-Cusato et al., 2004). A study using 129/ReW1xC57BL/6JWe mice demonstrated that prevention of the PD 0 T surge by neonatal castration led to significantly reduced mounting and intromission behavior compared to intact males whereas motivation appeared to be spared, based on the finding of similar mount latencies, indicating that the prenatal T surge is sufficient to organize male sex behavior while the postnatal T surge is required for maturation and refinement of male sex behavior (Quadagno et al., 1975). The extent to which perinatal gonadal steroids play an organizational role in sexual differentiation of the neural circuits controlling steroid-independent male sex behavior warrants further investigation.

Our analyses of male sex behavior after hormone replacement in adulthood in hybrid male mice that were gonadectomized prior to puberty revealed that estradiol treatment was significantly more effective than T in activating male sex behavior. Differential sensitivity to EB and T was evident on the initial behavioral test after one week of hormone treatment, and male sex behavior of prepubertal castrates treated with EB in adulthood was indistinguishable to that of hybrid non-maters treated with T (POSTPUB-GDX + T_{adult}) across all three hormone-replacement tests (Fig. 3A–C). Only after an additional week of hormone treatments with T did levels of activated mounting or intromitting behavior of the PREPUB-GDX + T_{adult} males reach equivalent levels of those treated with EB. Finally, relative to EB-treated animals, T-treated animals required 3 weeks of treatment before reaching the level of ejaculatory behavior displayed by EB-treated animals.

That an estrogen and not an androgen was able to activate male sex behavior after one week of hormone treatment is intriguing and suggests either a potential deficiency of androgen sensitivity or alternatively, an increased sensitivity to estrogens at the onset of hormone exposure. A reduced hypothalamic expression of aromatase may explain the reduced behavioral sensitivity of prepubertal castrated hybrid males to T. Estrogen signaling plays a major role in activating male sex behavior in mice (Ogawa et al., 2000); thus a potentially diminished aromatase activity may have rendered the T treatment less effective than EB because of the failure to convert a sufficient quantity of T into E2, which in turn failed to sufficiently activate hypothalamic estrogen receptors on the initial hormone replacement tests. Given the ability of estrogens to activate male sex behavior in multiple species, the relationship between aromatase activity and male sex behavior has been investigated in Japanese quail, a species which exhibits tight coupling between T levels and preoptic aromatase activity, with castration inhibiting aromatase and T rescuing aromatase activity (Balthazart and Foidart, 1993).

Three weeks of hormone treatment in adulthood (T, EB, or oil) was insufficient to lead to differences in AGD in male mice that were gonadectomized prior to puberty; however, the absence of gonadal steroids during puberty was sufficient to lead to decreased AGD relative to those that were deprived of gonadal steroids after puberty (Fig. 4). These results suggest that gonadal hormones may impact androgen-sensitive peripheral anatomy beyond the perinatal critical period, which corresponds with a prior study using Wistar rats (Kita et al., 2016).

Individual differences in sexual responsiveness of B6D2F1 castrates may not only reflect the high degree of individual genetic variation inherent to first generation hybrid mice created by crossing two inbred strains (Park et al., 2010), but also environmental variables. Several influencing factors such as maternal behavior, stress, intrauterine position, or endocrine-disrupting chemical exposure (Gore et al., 2015; Ryan and Vandenbergh, 2002; Ward and Ward, 1985) may significantly alter the normal processes of masculinization and defeminization, secondary to alterations in the level of circulating gonadal steroids or sex steroid receptor signaling cascades during the perinatal and peripubertal organizational periods during development (Ward and Weisz, 1980, 1984).

Because castration does not affect all androgen-dependent traits or all genotypes equally (Dark et al., 1987; Matochik and Barfield, 1994), whether all gonadally-mediated traits are retained to a greater extent in maters than in non-maters remains to be determined, as well as the extent to which each of those traits are organized by gonadal steroids during the perinatal and pubertal developmental periods. Increased aggressive behavior after gonadal regression has been documented in Siberian hamsters (Jasnow et al., 2000), and we are currently investigating whether B6D2F1 hybrid maters demonstrate steroid-independent aggression.

In summary, our results suggest that gonadal hormones, specifically T, secreted during puberty may play a significant role in long-lasting organizational change in the neural circuitry underlying several components of steroid-independent male sex behavior. Additionally, differential sensitivity of male sex behavior to activation by estrogens and androgens has been uncovered in B6D2F1 hybrid male mice that fail to demonstrate steroid-independent male sex behavior after long-term orchidectomy. Future studies of B6D2F1 hybrid maters stand to increase not only our understanding of the mechanisms by which environmental factors, sensory systems, and neurotransmitters sustain steroid-independent male sex behavior, but also the mechanisms by which gonadal hormones organize the neural underpinnings regulating steroid-independent male sex behavior during both the perinatal and peripubertal organizational periods.

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Declarations of interest

None.

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