Excess neonatal testosterone causes male-specific social and fear memory deficits in wild-type mice

Pravda Quiñones-Labernik[a], Kelsey L Blocklinger[b], Matthew R Bruce[c], Sarah L Ferri[d]*

[a]Department of Neuroscience and Pharmacology, University of Iowa, Iowa City, IA, United States
[b]Carver College of Medicine, University of Iowa, Iowa City, IA, United States
[c]Temple University, Philadelphia, PA, United States
[d]Department of Pediatrics, University of Iowa, Iowa City, IA, United States
*corresponding author sarah-ferri@uiowa.edu

Abstract

Neurodevelopmental disorders (ND) disproportionately affect males compared to females, and Autism Spectrum Disorder (ASD) in particular exhibits a 4:1 male bias. The biological mechanisms of this female protection or male susceptibility have not been identified. There is some evidence to suggest that fetal/neonatal gonadal hormones, which play pivotal roles in many aspects of development, may contribute. Here, we investigate the role of testosterone administration during a critical period of development, and its effects on social approach and fear learning in C57BL/6J wildtype mice. Male, but not female mice treated with testosterone on the day of birth (PN0) exhibited deficits in both social behavior and contextual fear conditioning, whereas mice treated with the same dose of testosterone on postnatal day 18 (PN18) did not display such impairments. Testosterone administration did not induce anxiogenic effects or lead to changes in body weight compared to the vehicle-treated group. These impairments are relevant to ND and may help identify novel treatment targets.

Keywords
Testosterone; Social behavior; Fear conditioning; Neurodevelopmental disorders; Autism; Sex differences

Introduction

A number of neurodevelopmental and neuropsychiatric disorders affect males and females divergently. In terms of prevalence, Autism Spectrum Disorder (ASD), Attention Deficit Hyperactivity Disorder (ADHD), and psychopathy affect males at a ~4, 2, and 2.7 to 1 ratio, respectively (1–3). Other factors such as age of onset, severity, and response to treatment can also look different across the sexes. For example, males tend to be diagnosed with schizophrenia at a younger age, experience more negative symptoms, exhibit higher levels of social isolation and substance use disorders, greater brain abnormalities, and show reduced responsiveness to antipsychotics compared to females (4–6). Women are more than twice as likely to develop post-traumatic stress disorder (PTSD) following trauma and tend to be more responsive to treatment than men (4). Behavioral symptoms and neurobiological profiles also differ between males and females diagnosed with ASD and ADHD (7,8). These neurodevelopmental and mental health conditions are increasing in prevalence, are difficult to treat, and can be emotionally, physically, and financially taxing not only to affected individuals, but also to their families and society (9,10). Therefore, it is crucial to determine the developmental processes impacting resilience and vulnerability to, and disease trajectories of, neurodevelopmental and neuropsychiatric disorders.

The mechanisms underlying the sex differences in neuropsychiatric conditions are not fully understood, and they are likely complex and multifactorial. However, one of the earliest developmental processes that may be involved is gonadal hormone exposure during the prenatal and neonatal periods. Human males experience a surge of testosterone in mid-gestation and shortly after birth, while male rodents have highest levels several days before and after birth. Females of those species are exposed to significantly lower levels of gonadal hormones during early development (11–13). In rats, many important and irreversible sex hormone-mediated effects on brain development will be complete by postnatal day 10 (14,15). After this period, sex hormones have less profound, more transient effects in the brain.

Studying the role of fetal and neonatal gonadal hormones in the development of neuropsychiatric disorders has proven challenging. Obvious ethical constraints prohibit well-controlled manipulations in humans and obtaining samples at the critical early timepoints is rare and problematic. The data we have is valuable but often confusing due to heterogeneity in human subjects and samples, lack of information (e.g. quantification of hormone levels *in utero*, measures of comorbidities, maternal stress, diet, etc.), and small sample size. For example, there has been conflicting evidence that androgens early in development contribute to the pronounced male sex bias in ASD. Using either direct measures in amniotic fluid and cord blood, or indirect measures, such as digit ratio (2D:4D) as an approximation for fetal testosterone exposure, some studies have found increased levels in children later diagnosed with ASD compared to typically developing children, while some have not (16–24). Other studies have shown that increased levels of prenatal testosterone correlate with increased autism trait scores in children without a diagnosis, but again, not all are in agreement (25–30).

Low 2D:4D ratio, indicating exposure to higher levels of testosterone prenatally, has also been associated with psychopathic characteristics (31,32). Studies investigating digit ratios in children with ADHD and anxiety disorders have yielded similar results, with many, but not all, finding evidence for increased prenatal testosterone relating to increased ADHD traits or symptom severity (8,33,34). Conversely, schizophrenia research has indicated that lower prenatal testosterone is a risk factor and sex steroids may play an important role in symptom manifestation (35,36). Therefore, while interpretation of these findings is complex, it seems clear that the role of developmental exposure to steroid hormones in later diagnosis warrants further

research. For this purpose, rodents are a valuable experimental model in which hormone manipulation during a critical period of development is well-controlled in timing, consistent and causal, and allows clearer interpretation of behavioral and functional results. Indeed, early postnatal testosterone administration to male rats with behavioral deficits relevant to ADHD resulted in exacerbation of impairments and changes in tyrosine hydroxylase expression (37,38). Here we used testosterone administration in mice on the day of birth to study behaviors that are disrupted in neurodevelopmental and neuropsychiatric disorders.

Many sexually divergent neuropsychiatric conditions present with changes in emotional response and involve impairments in approach and avoidance behaviors, including sociability and fear. One of the core symptoms of ASD is social impairments, but social behavior is also disrupted in ADHD, schizophrenia, PTSD, psychopathy, and numerous others (9,39–43). Many social behaviors exhibit sex differences and are sensitive to gonadal hormones as well as endocrine-disrupting agents (44). Acquisition of conditioned fear and fear extinction is impaired in several disorders including psychopathy, PTSD, and anxiety disorders (45,46). The ability to interpret both social and fear cues are critically important for physical and psychological well-being across many species. In the present study we sought to determine the effects of steroid hormone dysregulation on social approach and fear memory behaviors, which are relevant to neurodevelopmental and neuropsychiatric disorders. We administered a single dose of testosterone shown to masculinize a female rodent brain (100 µg, s.c.) to male and female C57BL/6J pups on the day of birth, which is during a critical period of sex-specific brain organization (14,15,47–51). We tested the mice in the three-chamber social approach assay as juveniles (postnatal day (PN) 28-32) and in contextual fear conditioning as adults (PN 53-64). **Fig. 1** provides an experimental overview. We found that a single administration of testosterone during a critical period of brain development (PN0), but not after (PN18), resulted in male-specific deficits in social approach and contextual fear conditioning that were not driven by changes in body weight or anxiety-like behavior.

Results

A single testosterone treatment on the day of birth results in male-specific social approach deficits in juveniles

We first aimed to determine whether excess neonatal testosterone could induce deficits associated with male-biased neurodevelopmental disorders, beginning with social approach. A preference index (PI) was calculated for each phase: (time spent sniffing social cylinder (empty in habituation or containing novel mouse during choice phase) – time spent sniffing nonsocial cylinder (empty or novel object)) / (total sniffing time). For the habituation phase, a two-way ANOVA revealed no main effect of sex ($F_{(1,65)}$=0.200, p=0.656, η^2=0.003), treatment ($F_{(1,65)}$=8.17, p=0.370, η^2=0.012), or sex x treatment interaction ($F_{(1,65)}$=0.034, p=0.855, η^2=0.0005; **Fig. 2A**). A two-way ANOVA of the PI in the choice phase uncovered no main effect of sex ($F_{(1,65)}$=2.188, p=0.144, η^2=0.028), a main effect of treatment ($F_{(1,65)}$=5.743, p=0.019, η^2=0.073), and a statistical trend of a sex x treatment interaction ($F_{(1,65)}$=3.827, p=0.055, η^2=0.049). A Tukey post hoc test indicated that males treated on PN0 with testosterone (Males + T) had significantly lower PI than Males + Veh, Females + Veh, and Females + T (p=0.013, 0.039, and 0.038, respectively; **Fig. 2B**).

Neonatal testosterone treatment had no effects on distance traveled during the social approach test. In juvenile male mice (28-32 d) treated with testosterone at PN0, a two-way ANOVA revealed no main effect of sex ($F_{(1,65)}$=0.355, p=0.553, η^2=0.005) or treatment ($F_{(1,65)}$=2.008, p=0.161, η^2=0.029), and no significant interaction during the habituation phase ($F_{(1,65)}$=2.655, p=0.108, η^2=0.038; **Fig. 2C**). Similarly, during the choice phase, there was no main effect of sex

($F_{(1,65)}$=1.029, p=0.314, η^2=0.015) or treatment ($F_{(1,65)}$=0.823, p=0.368, η^2=0.012), and no significant sex x treatment interaction ($F_{(1,65)}$=0.697, p=0.407, η^2=0.010; two-way ANOVA; **Fig. 2D**). Therefore, the decrease in social approach in juvenile males treated neonatally with testosterone was not an effect of altered locomotor behavior.

A single testosterone treatment on the day of birth results in male-specific contextual fear conditioning deficits in adults

After initial data collection revealed a male-specific social deficit, we used subsequent animals for contextual fear conditioning. Therefore, a subset of animals treated with veh or T on PN0 then tested for social approach as juveniles (28-32 d) additionally underwent 24-hr fear memory testing as adults (53-64 d). Percent time spent freezing during the pre-shock baseline period was similar across groups; a two-way ANOVA showed no main effect of sex ($F_{(1,40)}$=0.199, p=0.658, η^2=0.005), treatment ($F_{(1,40)}$=2.067, p=0.158, η^2=0.047), or sex x treatment interaction ($F_{(1,40)}$=1.372, p=0.248, η^2=0.031; **Fig. 3A**). A two-way ANOVA of freezing during the 24 h memory test uncovered a significant main effect of sex ($F_{(1,40)}$=4.514, p=0.040, η^2=0.087), treatment ($F_{(1,40)}$=4.310, p=0.044, η^2=0.084), and interaction ($F_{(1,40)}$=4.269, p=0.045, η^2=0.082). A Tukey post hoc test indicated that males treated on PN0 with testosterone (Males + T) had significantly lower freezing than Males + Veh, Females + Veh, and Females + T (p=0.029, 0.034, and 0.035, respectively; **Fig. 3B**).

Social and fear memory deficits are not due to differences in body weight or anxiety-like behavior

After determining that excess neonatal testosterone induces social and fear memory deficits in males only, we used separate groups of animals to determine whether our observed deficits may be due to changes in body weight or anxiety. Mice that underwent treatment with neonatal testosterone had similar body weight to those treated with veh on the day of birth (**Fig. 4A**). A RM three-way ANOVA revealed main effects of age ($F_{(2,60)}$=2525, p<0.0001, η^2=0.886), sex ($F_{(1,29)}$=12.69, p=0.001, η^2=0.004), and treatment (veh vs T; $F_{(1,29)}$=7.345, p=0.011, η^2=0.002), and the following significant interactions: age x sex ($F_{(8,232)}$=41.04, p<0.0001, η^2=0.014), age x treatment ($F_{(8,232)}$=4.967, p<0.0001, η^2=0.002), and age x sex x treatment ($F_{(8,232)}$=2.556, p<0.011, η^2=0.0008), but no sex x treatment interaction ($F_{(1,29)}$=0.027, p=0.871, η^2=7.9e-6). A Tukey post hoc test indicated that at age PN30, males treated with vehicle weighed significantly more than females treated with testosterone (p=0.021), and at PN 60, Males + Veh weighed significantly more than both Females + Veh and Females + T (p<0.0001 for both). In summary, most significant differences in weight were due to sex as expected, but there were no significant differences driven by testosterone within sexes at any age.

Naïve adult mice treated on the day of birth with testosterone did not show any differences in distance traveled or percent time spent in open arms in the elevated zero maze compared to those treated with vehicle on the day of birth (**Fig 4B and 4C**). A two-way ANOVA of total distance traveled over the 5-min test revealed no main effect of sex ($F_{(1,34)}$=0.102, p=0.751, η^2=0.003) or treatment ($F_{(1,34)}$=0.012, p=0.913, η^2=0.0003) and no interaction between the two ($F_{(1,34)}$=0.623, p=0.435, η^2=0.018). A two-way ANOVA of percent time spent in the open arms also revealed no differences, with no main effect of sex ($F_{(1,34)}$=3.117, p=0.087, η^2=0.08) or treatment ($F_{(1,34)}$=0.551, p=0.463, η^2=0.014) and no interaction between the two ($F_{(1,34)}$=2.090, p=0.158, η^2=0.054).

A single testosterone treatment on PN18 does not induce social approach deficits in juveniles

Next, we wanted to determine if the same dose of testosterone treatment given later, outside the reported critical period of brain masculinization (14,15), would cause similar social impairments. For PI during the habituation phase of juvenile mice treated with veh or

testosterone on PN18, a two-way ANOVA showed no main effects or interaction ($F_{(1,39)}$=0.010, p=0.922, η^2=0.0002 for sex; $F_{(1,39)}$=1.280, p=0.265, η^2=0.031 for treatment; $F_{(1,39)}$=0.586, p=0.449, η^2=0.014 for interaction; **Fig. 5A**). During the choice phase of the social approach test, all groups had a similar PI; a two-way ANOVA produced no main effects of sex ($F_{(1,39)}$=0.084, p=0.773, η^2=0.022) or treatment ($F_{(1,39)}$=0.001, p=0.971, η^2=0.00003), and no interaction of the two ($F_{(1,39)}$=0.120, p=0.731, η^2=0.003; **Fig. 5B**).

For distance traveled in the habituation phase, a two-way ANOVA revealed no main effect of sex ($F_{(1,39)}$=0.153, p=0.698, η^2=0.004), treatment ($F_{(1,39)}$=0.089, p=0.767, η^2=0.002), or sex x treatment interaction ($F_{(1,39)}$=0.345, p=0.561, η^2=0.009; **Fig. 5C**). A two-way ANOVA of the distance traveled in the choice phase uncovered a significant main effect of sex ($F_{(1,39)}$=8.207, p=0.007, η^2=0.167), no main effect of treatment ($F_{(1,39)}$=0.105, p=0.019, η^2=0.002), and no sex x treatment interaction ($F_{(1,39)}$=0.286, p=0.596, η^2=0.005). A Tukey post hoc test indicated that males treated on PN18 with testosterone (Males + T) had significantly lower distance traveled than Females + T (p=0.038; **Fig. 5D**), but there were no other group differences.

A single testosterone treatment on PN18 does not induce contextual fear conditioning deficits in adults

Similarly, a two-way ANOVA of percent time spent freezing during the baseline period demonstrated a trend toward a main effect of sex ($F_{(1,47)}$=3.887, p=0.055, η^2=0.074), no main effect of treatment ($F_{(1,47)}$=0.779, p=0.382, η^2=0.015), and no significant phase x sex interaction ($F_{(1,47)}$=0.081, p=0.778, η^2=0.031; **Fig. 5A**). Twenty-four hours later, freezing percentages were similar across all groups. A two-way ANOVA showed no significant main effect of sex ($F_{(1,47)}$=2.513, p=0.120, η^2=0.049) or treatment ($F_{(1,47)}$=0.605, p=0.441, η^2=0.012), and no interaction ($F_{(1,47)}$=1.421, p=0.239, η^2=0.028).

Discussion

Our data show that a single testosterone treatment on the day of birth results in both social deficits in juvenile, and fear memory deficits in adult, male wild-type (C57BL/6J) mice but has no effect on females. The same single s.c. injection of testosterone propionate (100 µg) (47–51) administered on postnatal day 18 has no effect on social or fear memory behavior at 30 or 60 d, respectively. Considering that male rodents exhibit peak levels of testosterone several days before and after birth, while females experience negligible exposure to gonadal hormones during early development, postnatal day 18 was chosen as a timepoint outside the endogenous hormone activity (14,15). Comparison at these two timepoints allows investigation of developmental time windows during which neural circuits underlying sociability and fear conditioning behaviors are more susceptible to sex-specific disruption. Our results confirm that excess testosterone is not always harmful but can disrupt sex-specific neural circuits during a critical organizational developmental period. Additionally, these findings indicate that both social approach behavior and fear memory exhibit sex differences in vulnerability in early development, which has significant implications for neurodevelopmental and neuropsychiatric disorders that affect males and females differently in terms of prevalence or progression. Importantly, the observed deficits were not due to changes in body weight or motor activity. The deficits were also not attributed to heightened anxiety-like behavior, which can co-occur with social deficits (52,53).

Notably, neonatal testosterone treatment did not affect litter size, pup mortality, or sex distribution (data not shown). Additionally, veh-treated mice exhibited behavior statistically equivalent to that of mice that were undisturbed as neonates (no neonatal injection); both social

preference indices at ~PN30 and 24 hr fear memory results at ~PN60 were comparable between mice administered veh and never-injected control mice we have collected over various experiments (54–57). However, there are several caveats to consider, including possible effects of neonatal injections on maternal care, which could in turn affect pup behavior (44,58). While we did not detect any differences in behavior of veh animals compared to other uninjected controls and the injection involved only a very brief (<2 min) separation from the dam, we are not able to fully rule out changes in the dam's response to the pups. In particular, licking and grooming may be increased immediately following the injection. This will be important to investigate in future experiments. An additional caveat is possible litter effects, in which high variance is observed across litters (59). In the present report, we used individual mice for each data point, rather than a litter average because each individual pup was administered treatment directly, rather than the dam being treated. However, we also included an average PI or percent time freezing, denoted by a yellow dot on each graph, to demonstrate values where n represents a litter rather than a single mouse, to show that it does not appear that any of our results were driven by litter effects. Relatedly, we treated the entire litter with veh or testosterone and same-treatment mice remained housed together, but it will be valuable to address whether behavioral effects would be affected if litters consisted of mixed-treatment mice and to explore litter effects more carefully. Additionally, we did not account for social dominance or hierarchical status, which can affect behavior, although stability of social ranking and under which specific circumstances it affects behavior are complex (60–64). This will be interesting to investigate in future experiments. However, historically, we have acquired very consistent, reliable, and robust social behavior in the brief and low-stress assay across differing social ranks and robust, reproducible fear memory as well, although those mice are single housed for several days prior to testing. We also expect that animals of differing social ranking would be randomly and equally distributed across both experimental groups.

An as-yet unknown factor is the circulating levels of testosterone following our treatment. Arguably, male mice treated with testosterone on postnatal day 0 are likely subjected to the additive effects of both endogenously secreted and exogenously administered hormones, but it is possible that compensatory or other unexpected mechanisms are involved. One study that used the same dose of testosterone as in our study, although it was administered on PN2, found changes in gene expression in the brains of PN6 males and females. Overall, 319 genes were differentially expressed between veh and T-treated males, and decreases in estrogen receptor- and androgen receptor-responsive gene elements were found in the flanking regions of those genes. Interestingly, levels of *Kiss1* (kisspeptin) gene expression were similar between females treated neonatally with testosterone and males treated with veh. Levels of *Kiss1*, *Esr2* (estrogen receptor β), and *Cyp19a1* (aromatase) were not statistically different in males treated with veh and males treated with T, but *Esr1* (estrogen receptor α; ERα) was downregulated in T-treated male brains (49). ERα-knockout mice exhibit social deficits and a single nucleotide polymorphism (SNP) was reported to correlate with severity in social interaction deficits in a group of children with autism, although ESR1 is not considered a high confidence risk gene for ASD (65–67). Therefore, the possibility of ESR1-mediated mechanism of approach and avoidance deficits will be important to study. While it seems that excess testosterone causes dysfunction sufficient to disrupt behavior, additional research is needed to directly measure the amount of exogenous testosterone reaching the brains of treated mice and to elucidate how exogenous administration affects the endogenous surge of testosterone and other downstream effects. Relatedly, it is difficult to determine how this amount of excess testosterone in neonatal mice compares to the slightly increased levels shown in human male fetuses that later went on to receive a diagnosis of ASD (18). Our treatment may very well be comparatively high, but our model provides a valuable opportunity to study developmental mechanisms that can affect sex-dependent vulnerabilities to impairments in important behaviors.

A critical next step is to determine the mechanisms by which excess testosterone on the day of birth induces male-specific social and fear deficits. Social behavior and fear conditioning both rely heavily on the medial prefrontal cortex (mPFC) and amygdala. Lesion and imaging studies have demonstrated the importance of the mPFC in social behaviors and fear conditioning in humans and rodents (68–77). Additionally, manipulation of various cell types and projection neurons in the mPFC can modulate social behaviors (78–83) and fear (84–86). Similarly, the amygdala has been labeled both a social hub and a major locus of fear conditioning. It is highly interconnected with many other brain areas important for these behaviors as well (77,87). Humans and animals with amygdala damage can exhibit impaired social behavior or fear conditioning deficits (76,77,88–92). fMRI studies in humans and electrophysiological studies in rodents also clearly demonstrate the involvement of various amygdalar nuclei in both behaviors (77,90,101,93–100). Likewise, immediate early gene expression, calcium imaging, and optogenetic and chemogenetic studies have solidified these findings (52,54,108–112,84,100,102–107). The hippocampus is also central to both behaviors, with the dorsal area more involved in fear learning (76,77,112–114), and the ventral with social behavior (115–118). The ventral tegmental area (VTA), nucleus accumbens (NAc), and cerebellum have also been implicated in the regulation of social behavior (39,119–122). Numerous sex differences in morphology and function in these brain regions have been documented, including in area volume, cell number, size, and structure, and most express androgen and estrogen receptors (123). Early testosterone-mediated effects may be acting in some of these brain areas to produce behavioral deficits.

Fetal/neonatal testosterone during the critical organizational period of brain development has important effects on a number of downstream processes. Neurotransmitter levels, receptor expression, neuropeptide signaling, neurogenesis, synaptic programming, and cell differentiation, migration, and death, are influenced by gonadal hormones during development and may be involved in the social and fear memory deficits (16,17,124,125). Investigating these potential mechanisms will provide insight into developmental processes involved in impairments associated with neurodevelopmental and other disorders. Another critical mechanistic question concerns the receptors that mediate the sex-specific impairments in behaviors related to approach and avoidance. Early in development, genes on the Y chromosome orchestrate the production of testosterone by the testis in males. Testosterone can then bind directly to or be metabolized into dihydrotestosterone, which binds to, androgen receptors, or it can be aromatized to estradiol and bind to estrogen receptors. Both processes are important for distinct components of brain masculinization and defeminization (11,13). It will be necessary to determine if either or both pathway(s) are disrupted to cause sex-specific social and fear deficits.

A final, significant point of clarification is whether excess testosterone in individuals is directly responsible for social and fear memory deficits and the neurodevelopmental and neuropsychiatric conditions that cause them. There are several circumstances in which a fetus may be exposed to excess testosterone, whether the source is the fetus, mother, or placenta (18,126). Congenital adrenal hyperplasia (CAH), Polycystic Ovarian Syndrome (PCOS), pre-eclampsia, and increased maternal psychological stress are conditions associated with increased androgen levels and have been associated with increased offspring risk for neurodevelopmental disorders (8,127–131). Therefore, hormone dysregulation alone, including testosterone but also other sex steroids, may be enough to induce related impairments in some, likely small, percentage of individuals. More likely however, is an interaction of genes, environment, and experiences that interact with sex-specific developmental pathways or one or

more of these factors acts in combination with hormone dysregulation to contribute to the development of these disorders (18,132,133).

Conclusions

Excess testosterone during a critical period of development induces long-term effects on social and fear memory behaviors in male mice. Importantly, we do not propose this manipulation as a model of any disorder; here we used neonatal hormone dysregulation to induce neurodevelopmentally relevant deficits as a first step towards investigation of sex-specific pathways that may confer vulnerability to disorders that exhibit sex bias.

Methods

Animals
Experiments were conducted in accordance with the University of Iowa Institutional Animal Care and Use Committee (IACUC) policies and ARRIVE guidelines, and mice were maintained consistent with the Guide for the Care and Use of Laboratory Animals. Mice were housed in groups of two to five per cage, unless otherwise specified, in a temperature and humidity controlled environment (22 °C and 55 ± 5%, respectively). All animals were housed on a 12-hour light/dark cycle. Food and water were available *ad libitum*. C57BL/6J (B6) mice were obtained from The Jackson Laboratories (#000664) to establish breeding cages which contained one dam and one sire. Litters were randomly divided into two experimental conditions: treatment with testosterone (T) or a vehicle (veh), receiving treatment on the day of birth (PN0) or on postnatal day 18 (PN18). Entire litters received the same treatment. On postnatal day 21 mice were weaned and housed with 2-5 same-sex littermates per cage. Gonadectomized A/J mice (#000646) were obtained from The Jackson Laboratories and were used as a social stimulus in the social behavioral assay. A total of 205 mice were used for behavioral testing. Of the 205 mice, 21 were excluded due to inactivity during the testing period, excessive climbing on cylinders, or statistical outliers (>2SD from the mean). Of mice treated neonatally, one cohort was used in weight studies, another to test anxiety-like behavior, and another in the social approach test, with a subset undergoing contextual fear conditioning, further outlined below. Mice treated on PN18 completed social approach and fear memory tests at PN 28-32 and 53-64, respectively, except for 7/51 mice underwent only fear conditioning as adults.

Testosterone Treatment
Testosterone propionate (Sigma; 100ug in 20 µl sesame oil, a dose previously shown to induce brain masculinization) or vehicle (20 µl sesame oil) was administered subcutaneously at one of two time points: to pups on the day of birth (PN0) or on postnatal day 18 (PN18) (47–51). Breeding cages were checked regularly for litters and pups were injected within 10 hours of birth. Neonates were separated from the dam for less than two minutes and gently scruffed for the subcutaneous injection. Following treatment, pups were returned to their homecage with the dam and sire.

Weight Measurement
In an independent cohort of animals, to avoid repeated handling prior to behavioral assays, weights were measured every two days, beginning on day 2 after birth (PN2), until day 12. Then, weights were recorded on day 21, 30, and 60 to monitor treatment effects.

Social Approach Test

The Social Approach Test was conducted in mice aged 28-32 days, under dark conditions, using a black Plexiglass arena that had three chambers devoid of top and bottom, which was placed on a clear Plexiglass table over a clean absorbent pad. Identical bottomless and topless clear cylinders were placed at the center of both outer arena chambers. Each clear cylinder featured one end with small breathing holes which facilitated air circulation and enabled visual and olfactory exploration. Flat lids were placed on top of the cylinders and secured with small paperweights. The testing room was dimly lit, and the chamber was illuminated from below using infrared light. Testing sessions were recorded from an overheard-positioned camera (Basler Ace GIGE). The behavioral assay consisted of two ten-minute phases: a “Habituation” phase followed by a “Choice” phase. During the habituation phase, the test mouse could freely explore the chamber and empty cylinders. Following the completion of the habituation phase, a novel-object (Duplo block) was introduced into one cylinder, while a novel social stimulus, a same-sex gonadectomized A/J mouse, was placed in the opposite cylinder. Again, the mouse was able to freely explore for 10 minutes during the choice phase. Distance traveled and duration of sniffing of each cylinder were quantified using Noldus EthoVision XT video tracking software. A preference index (PI) was calculated for each phase: (time spent sniffing social cylinder (empty in habituation or containing novel mouse during choice phase) – time spent sniffing nonsocial cylinder (empty or novel object)) / (total sniffing time). A preference index of 0 indicates no preference for either cylinder (equal sniffing of each) and PI of 1 indicates 100% sniffing of social cylinder in the choice phase (or empty cylinder in the habituation phase).

Contextual Fear Conditioning

Approximately 30 days after the social behavioral assay, a subset of mice underwent contextual fear conditioning testing (53-64 d). Mice were singly housed 4-7 days prior to conditioning and handled 2-3 min each for 3 consecutive days prior to the assay. On the day of training, each mouse was placed inside a chamber with electrified metal grid flooring (CleverSys) inside a sound-attenuating box (Med Associates) for a duration of 3 min. During the initial 2min and 28s, the mice were allowed to freely explore the chamber, which served as a “baseline” period. After this time, a 1.5mA footshock was delivered to the mice for 2s. The mice were removed 30s following the shock. The test session was conducted 24hr later, during which the mice were placed in the same chamber for a period of 5min. The Cleversys Freezescan software was utilized to record the freezing behavior of the mice.

Elevated Zero Maze

Cohorts of naïve adult male and female mice (62-96) were utilized to assess anxiety-like behavior in PN0 testosterone- and vehicle- treated mice using an elevated zero maze (EZM). The elevated zero maze is an elevated ring-shaped runway with two open arms and two opposing closed arms. The open arms are devoid of walls resulting in an exposed environment, while the closed arms are enclosed with walls. The elevated zero maze was positioned beneath a camera (Basler Ace GIGE) in 250lux lighting conditions. MediaRecorder software was used to record the trials. Mice were placed on a boundary between an open and closed area facing the closed area. Each mouse was given a 5-min trial, during which they were allowed to freely roam the maze. The experimenter positioned themself behind a white curtain throughout the trial. The maze was cleaned with paper towels and 70% ethanol between each trial. Noldus Ethovision XT video tracking software was used to analyze time spent in the open versus closed areas and total distance traveled within the arena.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 9. Weight data were analyzed used a repeated measures three-way ANOVA to determine main effects of time, sex, treatment, and interactions. For all other data, a two-way ANOVA was performed with sex and treatment as

main effects, and a sex x treatment interaction was also tested. A Tukey post hoc test was used when appropriate. Spearman's test was used to determine heteroscedasticity and Shapiro-Wilk for test of normality. Log transformation was used in case of violations, however, raw data is shown in the graph for clarity. Significance was set to $p<0.05$. Bar graphs and error bars represent mean ± SEM and individual data points are shown. Eta squared values were used for effect size estimations.

Data Availability

Raw and analyzed data are available upon request from the corresponding author.

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Author contributions
SLF designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. PQL performed the experiments, analyzed the data, and wrote the manuscript. KLB and MRB performed the experiments and analyzed the data.

Additional Information
The authors declare no competing interests.

Figure 1

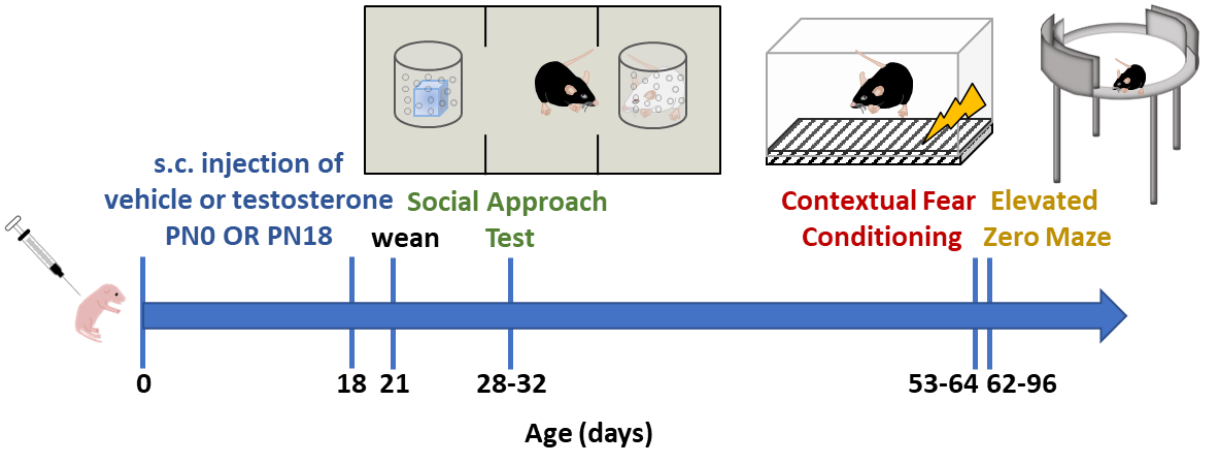

Figure 1. Experimental overview. Pups were injected subcutaneously on the day of birth (postnatal day 0) with testosterone or oil vehicle then remained with the dam and sire until weaning at PN 21, after which they were subjected to a weight study (not shown here, PN 2-60), social approach test at PN 28-32 and contextual fear conditioning at PN 53-64, or elevated zero maze at PN 62-96. Another group of mice was administered the same dose of testosterone or vehicle on PN 18, weaned at PN 21, and underwent social approach test at PN 28-32 and contextual fear conditioning at PN 53-64.

Figure 2

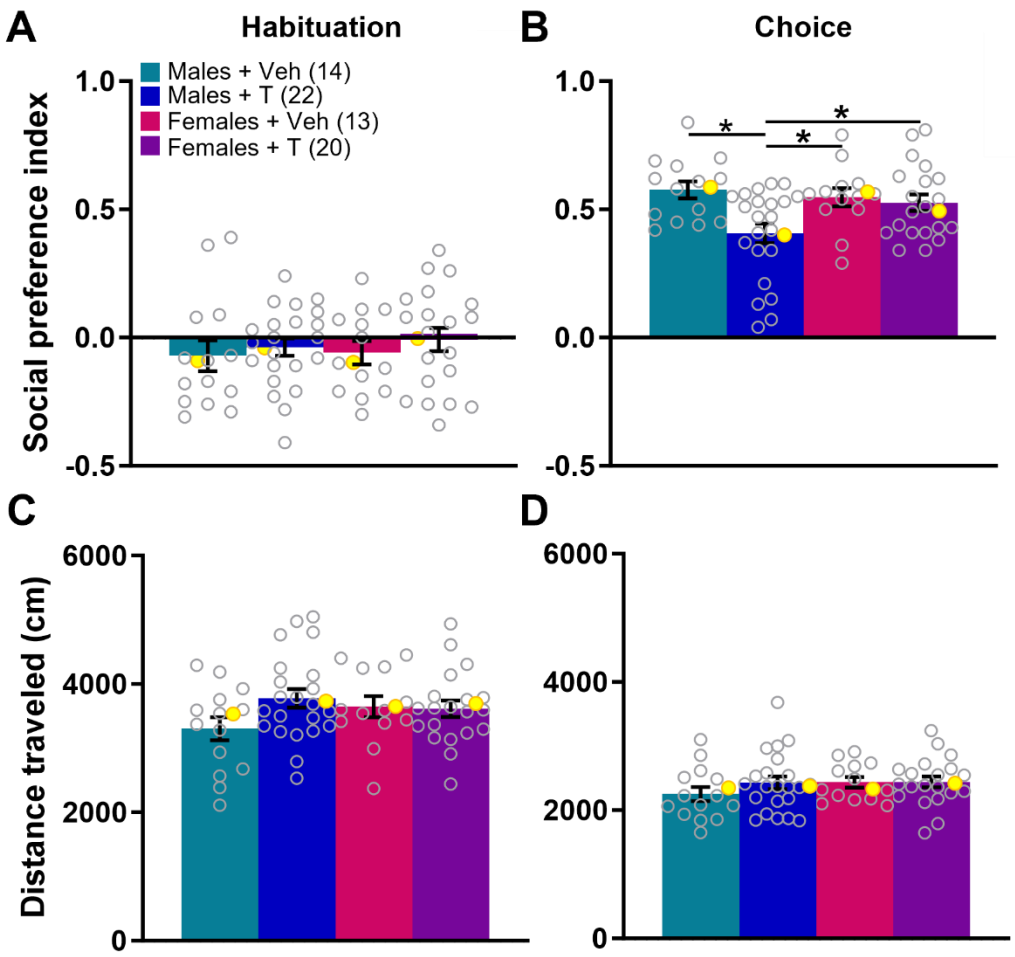

Figure 2. Testosterone administration on the day of birth induces social approach deficits in adolescent males. (A) During a 10 min habituation period of the social approach test, all experimental groups had a similar preference index (PI). (B) Males treated neonatally with testosterone had a significantly lower social preference index than males or females treated with vehicle or females treated with testosterone during the choice phase of the social approach test. There were no differences across groups in distance traveled during the social approach test in either the habituation (C) or choice phase (D).*$p<0.05$. Bars indicate mean ± SEM. Individual data points shown are individual mice and yellow dots indicate average litter values where each data point is a litter.

Figure 3

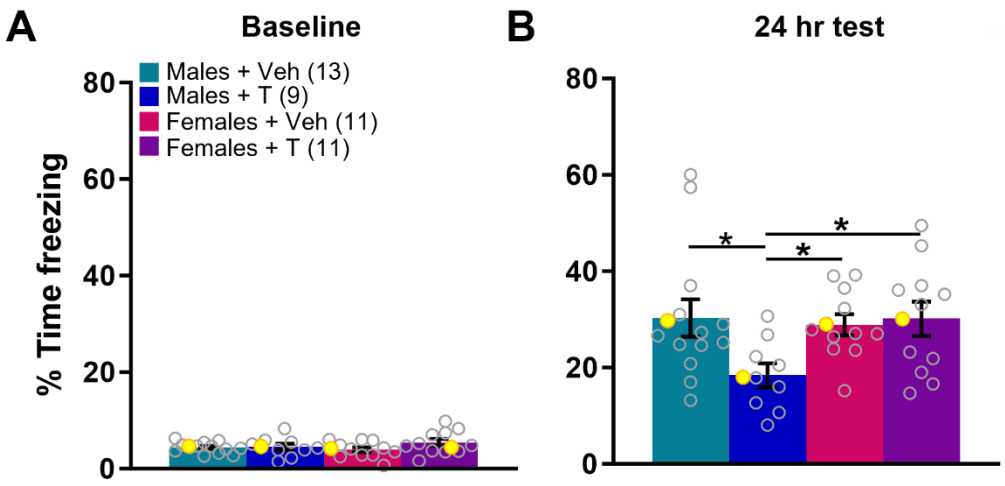

Figure 3. Testosterone administration on the day of birth induces fear memory deficits in adult males. (A) Baseline freezing prior to contextual fear conditioning was similar regardless of sex or treatment. (B) Adult males treated on the day of birth exhibited significantly less freezing during the 24 hr memory test than those treated with veh or females treated with either veh or T. *p<0.05. Bars indicate mean ± SEM. Individual data points shown are individual mice and yellow dots indicate average litter values where each data point is a litter.

Figure 4

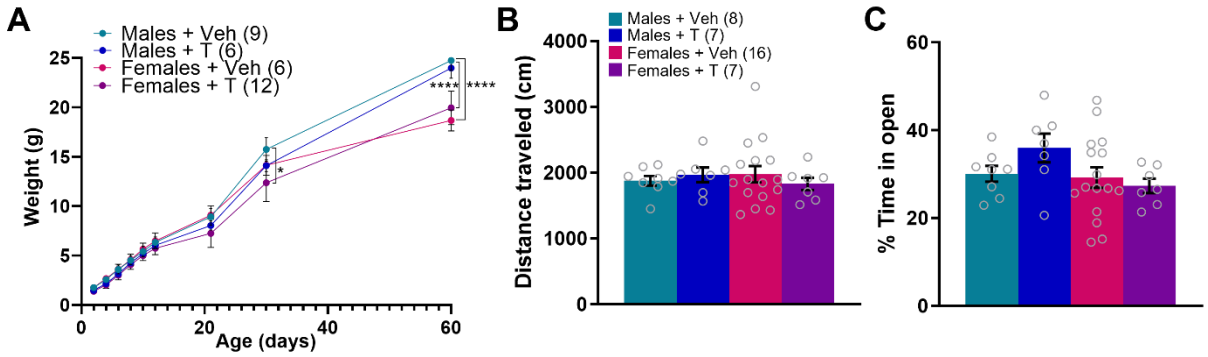

Figure 4. Excess neonatal testosterone-induced effects are not due to changes in weight or anxiety-like behavior. (A) Testosterone administration on the day of birth does not affect body weight. Mice treated at PN0 with testosterone or veh were weighed on PN 2, 4, 6, 8, 10, 12, 21, 30, and 60. Most differences in weight were driven by sex. Specifically, at PN30, males treated with vehicle weighed significantly more than females treated with testosterone and at PN 60, males treated neonatally with vehicle weighed significantly more than females treated either with vehicle or testosterone on the day of birth. (B) Naïve adult males and females treated neonatally with testosterone traveled similar distances in the elevated zero maze as those treated with veh. (C) Testosterone treatment on the day of birth had no effect on percent time spent in the open arms of the EZM. *$p<0.05$, ****$p<0.0001$.

Figure 5

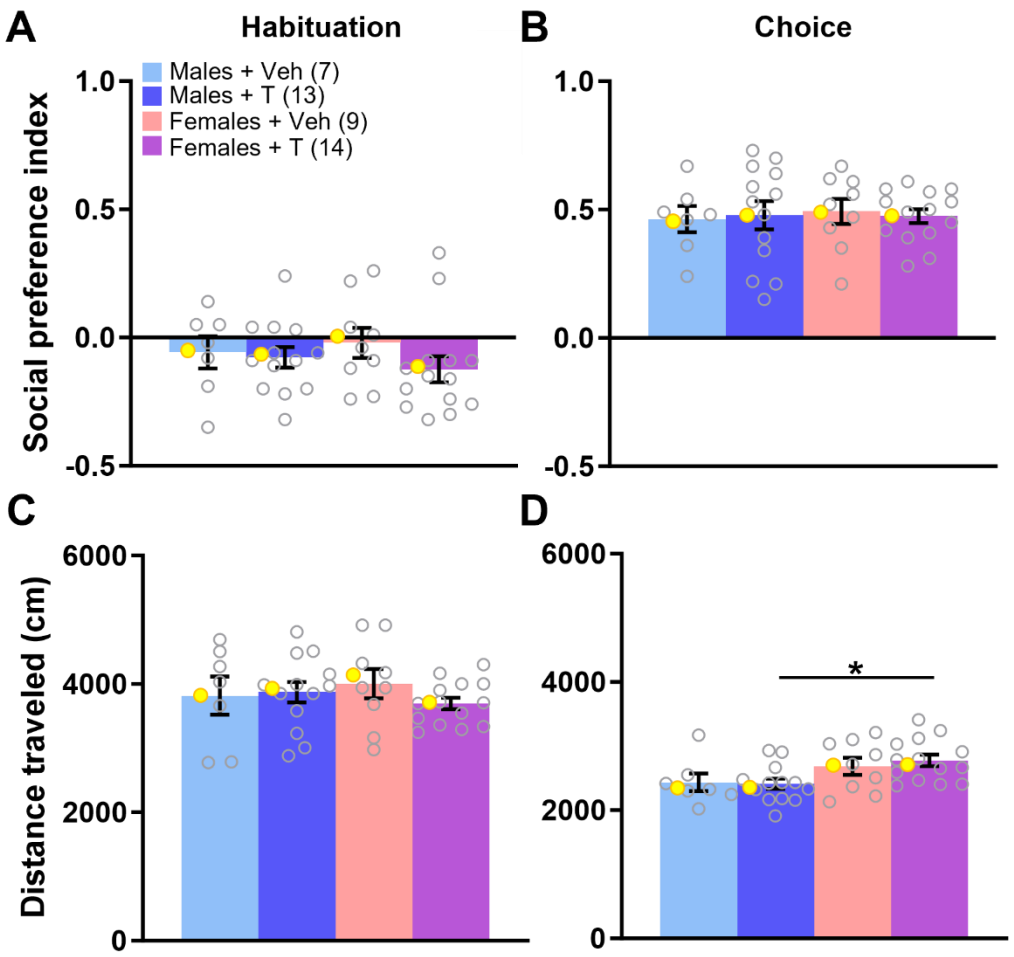

Figure 5. Testosterone administered on PN18 does not affect social approach behavior. (A) Male and female adolescent mice exhibited no significant differences in PI during habituation regardless of treatment with veh or T on PN 18. (B) Testosterone treatment on PN18 had no effect on PI during the choice phase. (C) During habituation, all experimental groups traveled similar distances. (D) Male + Veh animals traveled less than Female + T animals during the choice phase. *$p<0.05$. Bars indicate mean ± SEM. Individual data points shown are individual mice and yellow dots indicate average litter values where each data point is a litter.

Figure 6

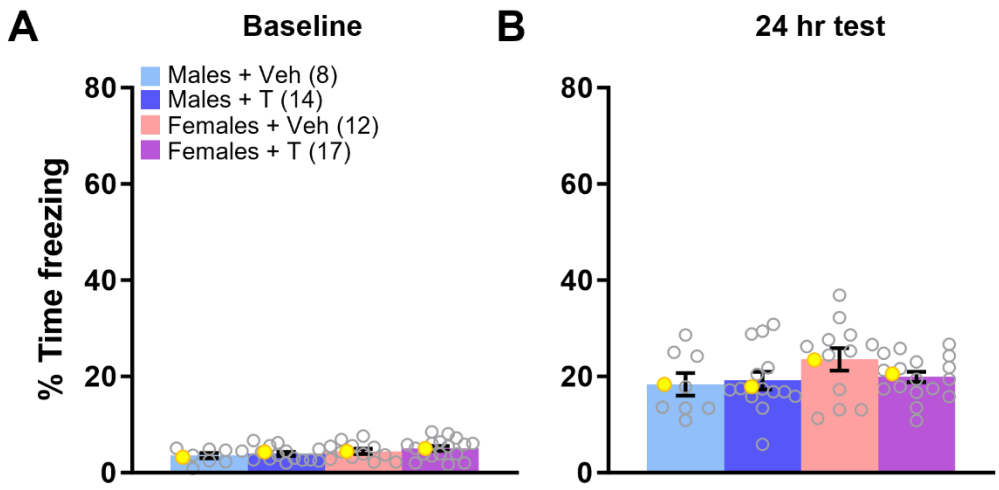

Figure 6. Testosterone administration on PN18 does not affect contextual fear conditioning in adults. (A) Treatment with T on PN18 did not affect percent of time spent freezing during the baseline measure. (B) There were no group differences in freezing during the 24 hr test of contextual fear memory. Bars indicate mean ± SEM. Individual data points shown are individual mice and yellow dots indicate average litter values where each data point is a litter.