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

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Highlights

- Male mice treated with testosterone at birth displayed reduced social approach behavior as juveniles and demonstrated impairments in contextual fear conditioning as adults, compared to mice treated with vehicle oil.
- Testosterone treatment on postnatal day 18 did not affect social approach or fear memory.
- This single dose of testosterone on PN0 did not induce anxiety-like behavior in testosterone-treated mice compared to vehicle-treated control mice.
- Neonatal testosterone administration did not result in a weight change compared to veh-treated mice.

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 weight compared to the testosterone-treated group. These impairments are relevant to ND and may help identify novel treatment targets.

Keywords

Testosterone; Gonadal hormones; Social behavior; Fear conditioning; Neurodevelopmental disorders; Autism; Sex differences

1. 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 (Maenner et al., 2023; Sanz-García et al., 2021; Yang et al., 2022). 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 (Ferrer-Quintero et al., 2021; Gogos et al., 2019; Li et al., 2016). 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 (Gogos et al., 2019). Behavioral symptoms and neurobiological profiles also differ between males and females diagnosed with ASD and ADHD (Davies, 2014; Santos et al., 2022). 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 (Homberg et al., 2016; Zablotsky et al., 2019). 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 exponentially lower levels of gonadal hormones during early development (Gillies and McArthur, 2010; Konkle and McCarthy, 2011; McCarthy, 2011). In rats, many important and irreversible sex hormone-mediated effects on brain development will be complete by postnatal day 10 (Davis et al., 1996; McCarthy et al., 2017). 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 do 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 (Al-Zaid et al., 2015; Baron-Cohen et al., 2015, 2005; Barona et al., 2015; Eriksson et al., 2016; Falter et al., 2008; Ferri et al., 2018; Guyatt et al., 2015; Whitehouse et al., 2010). 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 (Auyeung et al., 2010, 2009; Chapman et al., 2006; Knickmeyer et al., 2006; Kung et al., 2016; Lutchmaya et al., 2002).

Low 2D:4D ratio, indicating exposure to higher levels of testosterone prenatally, has also been associated with psychopathic characteristics (Blanchard et al., 2016; Perez et al., 2022).

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 (Davies, 2014; De Bruin et al., 2006; Lemiere et al., 2010). Conversely, schizophrenia research has indicated that lower prenatal testosterone is a risk factor and sex steroids may play an important role in symptom manifestation (Collinson et al., 2010; Markham, 2012). 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 (King et al., 2000; Li and Huang, 2006). 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 many others (Frye, 2018; Homberg et al., 2016; Kennedy and Adolphs, 2012; Nijmeijer et al., 2008; Porcelli et al., 2019; Scoglio et al., 2022). Acquisition of conditioned fear and fear extinction is impaired in several disorders as well, including psychopathy, PTSD, and anxiety disorders (VanElzakker et al., 2014; Veit et al., 2013). 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, anxiety-like, and fear memory behaviors, which are relevant to neurodevelopmental and neuropsychiatric disorders. A single administration of testosterone during a critical period of brain development resulted in male-specific deficits in social approach and contextual fear conditioning.

2. Materials and Methods

2.1 Animals

Experiments were conducted in accordance with the University of Iowa Institutional Animal Care and Use Committee (IACUC) policies 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 cohorts: a neonatal treated group, receiving treatment on the day of birth (PN0), and a group treated on postnatal day 18 (PN18). Mice were further subdivided into two experimental conditions: a testosterone (T)-administered cohort or a vehicle (veh)-administered cohort. On postnatal day 21 mice were weaned, 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.

2.2 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) (Hisasue et al., 2010; Seney et al., 2012). Following treatment, pups were reunited with their parents and returned to their original housing until the time of weaning.

2.3 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.

2.4 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).

2.5 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.

2.6 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 themselves 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.

2.7 Statistical Analysis

Statistical analysis was performed in GraphPad Prism 9. Analyses used a repeated measures three-way ANOVA to determine main effects of time or phase of test, sex, treatment, and interactions, except for EZM distance traveled and % time in the open, in which a two-way ANOVA was performed with sex and treatment as main effects. Tukey post hoc test was used when appropriate. 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.

3. Results

3.1 Neonatal testosterone does not significantly affect body weight

Mice that underwent treatment with neonatal testosterone had similar body weight to those treated with veh on the day of birth (**Fig. 1 and 2A**). A RM three-way ANOVA revealed main effects of age ($F_{(2,60)}=2525$, $p<0.0001$, $\eta^2=0.886$), sex ($F_{(1,29)}=12.69$, $p=0.001$, $\eta^2=0.004$), and treatment (veh vs T; $F_{(1,29)}=7.345$, $p=0.011$, $\eta^2=0.002$), and the following significant interactions: age x sex ($F_{(8,232)}=41.04$, $p<0.0001$, $\eta^2=0.014$), age x treatment ($F_{(8,232)}=4.967$, $p<0.0001$, $\eta^2=0.002$), and age x sex x treatment ($F_{(8,232)}=2.556$, $p<0.011$, $\eta^2=0.0008$), but no sex x treatment interaction ($F_{(1,29)}=0.027$, $p=0.871$, $\eta^2=7.9e-6$). 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.

3.2 Excess neonatal testosterone does not induce anxiety-like behavior

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 2B and 2C**). A two-way ANOVA of total distance traveled over the 5-min test revealed no main effect of sex ($F_{(1,34)}=0.023$, $p=0.881$, $\eta^2=0.0006$) or treatment ($F_{(1,34)}=0.044$, $p=0.835$, $\eta^2=0.001$) and no interaction between the two ($F_{(1,34)}=0.805$, $p=0.376$, $\eta^2=0.023$). A two-way ANOVA of percent time spent in the open arms also revealed no differences, with no main effect of sex ($F_{(1,34)}=3.117$, $p=0.087$, $\eta^2=0.08$) or treatment ($F_{(1,34)}=0.551$, $p=0.463$, $\eta^2=0.01$) and no interaction between the two ($F_{(1,34)}=2.090$, $p=0.158$, $\eta^2=0.054$).

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

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 RM three-way ANOVA revealed a main effect of phase (habituation vs choice; $F_{(1,65)}=386.5$, $p<0.0001$, $\eta^2=0.525$), but no main effect of sex ($F_{(1,65)}=0.701$, $p=0.406$, $\eta^2=0.003$) or treatment ($F_{(1,65)}=1.807$, $p=0.184$, $\eta^2=0.009$), and no significant interactions (phase x sex, $F_{(1,65)}=0.004$, $p=0.945$, $\eta^2=5.9e-6$; phase

x treatment, $F_{(1,65)}$=1.151, p=0.287, η^2=0.002; sex x treatment, $F_{(1,65)}$=2.121, p=0.150, η^2=0.010; phase x sex x treatment, $F_{(1,65)}$=1.971, p=0.165, η^2=0.003). Overall, mice traveled more during the habituation phase than the choice phase. A Tukey post hoc test indicated no differences in distance traveled within each phase (**Fig 3A**). A RM three-way ANOVA of the social preference index (PI) shows a significant main effect of phase (habituation vs choice $F_{(1,65)}$=219.6, p<0.0001, η^2=0.622), no main effect of sex ($F_{(1,65)}$=1.061, p=0.307, η^2=0.002), and a main effect of treatment ($F_{(1,65)}$=8.633, p=0.005, η^2=0.018). A sex x treatment interaction was also significant ($F_{(1,65)}$=9.534, p=0.003, η^2=0.020), while the following interactions were not statistically significant: phase x sex ($F_{(1,65)}$=1.018, p=0.317, η^2=0.003), phase x treatment ($F_{(1,65)}$=2.182, p=0.145, η^2=0.006), and phase x sex x treatment ($F_{(1,65)}$=0.191, p=0.664, η^2=0.0005). A Tukey post hoc test uncovered no group differences in the habituation phase, but in the choice phase, Males + T had a significantly lower social preference index than Males + Veh and Females + Veh, and there was a statistical trend compared to Females + T (p=0.004, 0.040, and 0.058, respectively; **Fig 3B**).

In contrast, animals treated with the same dose of testosterone on PN18 did not show any treatment-related effects on distance traveled or preference index when tested in the social approach test as juveniles (28-32 days old) (Fig **3C** and **3D**). For distance traveled, a RM three-way ANOVA revealed a main effect of phase (habituation vs choice; $F_{(1,39)}$=219.6, p<0.0001, η^2=0.611), no main effect of sex ($F_{(1,39)}$=0.992, p=0.325, η^2=0.006) or treatment ($F_{(1,39)}$=0.005, p=0.941, η^2=3.14e-5), and a significant phase x sex interaction ($F_{(1,39)}$=5.146, p=0.029, η^2=0.014). There were no significant interactions between phase and treatment ($F_{(1,39)}$=0.277, p=0.602, η^2=0.0008), sex and treatment ($F_{(1,39)}$=0.037, p=0.849, η^2=0.0002), and phase, sex, and treatment ($F_{(1,39)}$=0.939, p=0.339, η^2=0.003). A Tukey post hoc test did not reveal any differences between groups in either the habituation or choice phase. Overall, mice traveled more during the habituation phase than the choice phase. Similarly, a RM three-way ANOVA of preference index data demonstrated a main effect of phase (habituation vs choice; $F_{(1,39)}$=117.3, p<0.0001, η^2=0.603), no main effect of sex ($F_{(1,39)}$=1.156, p=0.289, η^2=0.005) or treatment ($F_{(1,39)}$=0.392, p=0.535, η^2=0.002), and no significant phase x sex ($F_{(1,39)}$=0.232, p=0.633, η^2=0.001), phase x treatment ($F_{(1,39)}$=0.367, p=0.548, η^2=0.002), sex x treatment ($F_{(1,39)}$=0.063, p=0.804, η^2=0.0002), or phase x sex x treatment ($F_{(1,39)}$=0.589, p=0.448, η^2=0.003) interactions. A Tukey post hoc test indicated that all experimental groups exhibited similar social preference index values in both the habituation and choice phases.

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

Adult mice (53-64 d) that underwent contextual fear conditioning exhibited male-specific deficits if they were administered testosterone on PN0 (**Fig 4A**) but not PN18 (**Fig 4B**). In PN0-treated mice trained with one 1.5 mA shock and tested in the same context, a RM three-way ANOVA revealed a main effect of session (baseline vs 24 hr test; $F_{(1,40)}$=182.7, p<0.0001, η^2=0.624), no main effect of sex ($F_{(1,40)}$=2.589, p=0.116, η^2=0.009) or treatment ($F_{(1,40)}$=1.800, p=0.187, η^2=0.006), and no significant session x sex ($F_{(1,40)}$=2.213, p=0.145, η^2=0.007), session x treatment ($F_{(1,40)}$=3.435, p=0.071, η^2=0.012), or session x sex x treatment ($F_{(1,40)}$=3.176, p=0.082, η^2=0.011) interactions. There was a significant sex x treatment interaction ($F_{(1,40)}$=4.577, p=0.039, η^2=0.016). A Tukey post hoc test showed no differences in baseline freezing. However, during the 24 hr test, Males + T spent a significantly lower percentage of time freezing than Males + Veh, and Females + T (p=0.015, 0.025, respectively), and trended towards significance compared to Females + Veh (p=0.066). In mice treated with vehicle or testosterone on PN18 in the same conditions, a RM three-way ANOVA similarly showed main effects of session (baseline vs 24 hr test; $F_{(1,47)}$=280.1, p<0.0001, η^2=0.690) and sex ($F_{(1,47)}$=4.108, p=0.048, η^2=0.011), but not treatment ($F_{(1,47)}$=0.258, p=0.614, η^2=0.0006). There

were no significant interactions (session x sex, $F_{(1,47)}$=1.033, p=0.315, η^2=0.003; session x treatment, $F_{(1,47)}$=0.996, p=0.323, η^2=0.002; sex x treatment, $F_{(1,47)}$=1.123, p=0.295, η^2=0.003; session x sex x treatment, $F_{(1,47)}$=1.540, p=0.221, η^2=0.004). Tukey multiple comparisons indicate that all groups spent a similar percentage of time freezing within the baseline period of training and within the 24 h test.

4. 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 (C57/BL6) mice but has no effect on females. Importantly, the same single s.c. injection of testosterone propionate (100 µg) (Hisasue et al., 2010; Seney et al., 2012) administered on postnatal day 18 has no effect on social or fear memory behavior at 30 or 60 d, respectively. These results confirm that testosterone administration is not universally damaging but can disrupt sex-specific neural circuits during an important 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 important 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 the result of increased anxiety-like behavior, which can co-occur with social deficits (Allsop et al., 2014; Felix-Ortiz et al., 2016).

An important next step is to determine the mechanisms by which excess testosterone on the day of birth induces male-specific social and fear deficits. Both social behavior and fear conditioning rely heavily on both 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 (Anderson et al., 1999; Barrash et al., 2010; Berthoz et al., 2002; Bicks et al., 2015; Eslinger et al., 2004; Forbes and Grafman, 2010; Frost et al., 2021; Kietzman and Gourley, 2023; Kim and Jung, 2006; Maren, 2001). Additionally, manipulation of various cell types and projection neurons in the mPFC can modulate social behaviors (Bicks et al., 2020; Cao et al., 2018; Ferenczi et al., 2016; Liu et al., 2020; Qin et al., 2019; Yizhar et al., 2011) and fear (Gilmartin et al., 2014; H. S. Kim et al., 2016; Luchkina and Bolshakov, 2019). 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 (Bickart et al., 2014; Kim and Jung, 2006). Humans and animals with amygdala damage exhibit impaired social behavior or fear conditioning (Adolphs, 2010; Amaral et al., 2003; Bliss-Moreau et al., 2013; Daenen et al., 2003; Kim and Jung, 2006; Machado and Bachevalier, 2006; Maren, 2001). fMRI studies in humans and electrophysiological studies in rodents also clearly demonstrate the involvement of various amygdalar nuclei in both behaviors (Adolphs, 2010; Bickart et al., 2011; Bucan et al., 2009; Davis et al., 2010; Hadjikhani et al., 2007; Katayama et al., 2009; Kim and Jung, 2006; Kleinhans et al., 2016; Kuga et al., 2022; Sato et al., 2020; Von der Heide et al., 2014). Likewise, immediate early gene expression, calcium imaging, and optogenetics and chemogenetics have solidified these findings (Felix-Ortiz et al., 2016; Felix-Ortiz and Tye, 2014; Ferri et al., 2016; Folkes et al., 2020; Jasnow et al., 2013; C. K. Kim et al., 2016; Kuga et al., 2022; LaLumiere, 2014; Luchkina and Bolshakov, 2019; Rogers et al., 2017; Sengupta et al., 2018; Siuda et al., 2016; Sych et al., 2018; Wei et al., 2023; Zaki et al., 2022). The hippocampus is also important for both behaviors, with the dorsal area more involved in fear learning (Anagnostaras et al., 2001; Kim and Jung, 2006; Maren, 2001; Marschner et al., 2008; Zaki et al., 2022), and the ventral with social behavior (Bannerman et al., 2002; Deng et al., 2019; Sams-Dodd et al., 1997; Sun et al., 2020). The ventral tegmental area (VTA), nucleus accumbens (NAc), and cerebellum have also been

implicated in the regulation of social behavior (Carta et al., 2019; Gunaydin et al., 2014; Musardo et al., 2022; Porcelli et al., 2019; Solié et al., 2022). Many of these brain areas are sexually dimorphic in terms of volume, cell number, size, or morphology, and most express androgen and estrogen receptors as well (Premachandran et al., 2020). Early testosterone-mediated effects may be acting in some of these brain areas to produce behavioral deficits.

Another important mechanistic question is what receptors may be mediating 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 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 (Gillies and McArthur, 2010; McCarthy, 2011). It will be important to determine if either or both pathway(s) are disrupted to cause sex-specific social and fear 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 (Baron-Cohen et al., 2011, 2005; Ferri et al., 2018; Schaafsma et al., 2017). Investigating these potential mechanisms will provide insight into developmental processes involved in impairments associated with neurodevelopmental and other disorders.

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 (Baron-Cohen et al., 2015; Van De Beek et al., 2004). 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 (Davies, 2014; Gumusoglu et al., 2020; Knickmeyer and Baron-Cohen, 2006; Kumar et al., 2018; Lai et al., 2011; Li et al., 2023). Therefore, hormone dysregulation alone 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 (Schaafsma and Pfaff, 2014).

5. Conclusions

Excess testosterone during a critical period of development induces lasting 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 significant deficits as a first step towards investigation of sex-specific pathways that may confer vulnerability to disorders that exhibit sex bias.

Acknowledgements
This work was supported by the National Institutes of Health (K01 MH119540) and University of Iowa Hawkeye Intellectual and Developmental Disabilities Research Center (Hawk-IDDRC; NICHD; P50 HD103556). We would like to thank Dr. Shane Heiney and the Neural Circuits and

Behavior Core at the University of Iowa and Emily Hagan, Danielle Preuschl, and Charlotte Tesar for their input and support.

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Figure 1

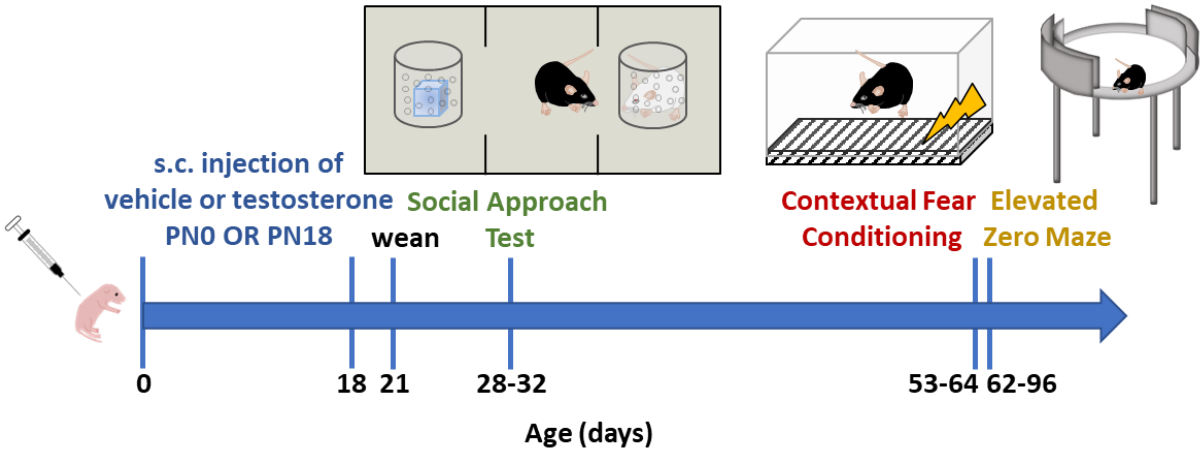

Figure 1. Experimental overview. Pups were injected subcutaneously on the day of birth (postnatal day 0) with testosterone or oil vehicle then remained with their mother and father until weaning at PN 21, after which they were subjected to a weight study (not shown here, PN 2-60), social approach test 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

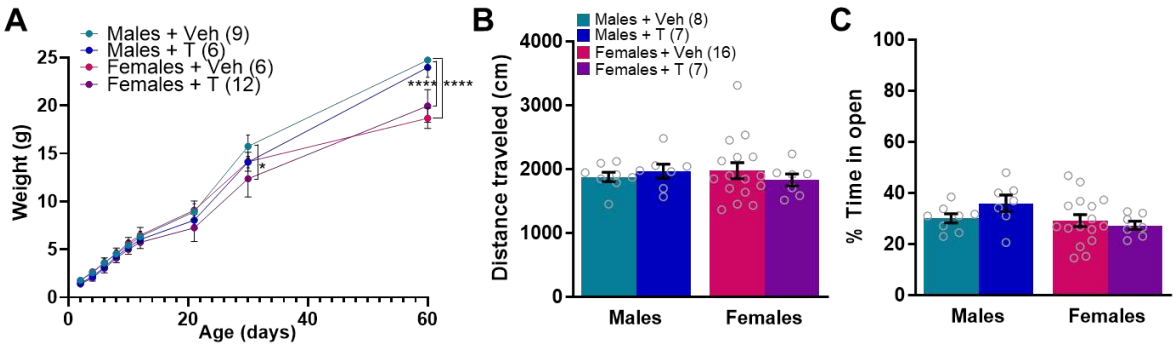

Figure 2. Testosterone administration on the day of birth does not affect body weight or anxiety-like behavior. (A) Mice treated at PN0 with testosterone or veh were weighed on PN 2, 4, 6, 8, 10, 12, 21, 30, and 60. Most differences in weight were driven by sex. Specifically, at PN30, males treated with vehicle weighed significantly more than females treated with testosterone (*p=0.021), 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 (****p<0.0001 for both). (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 open arms of the EZM.

Figure 3

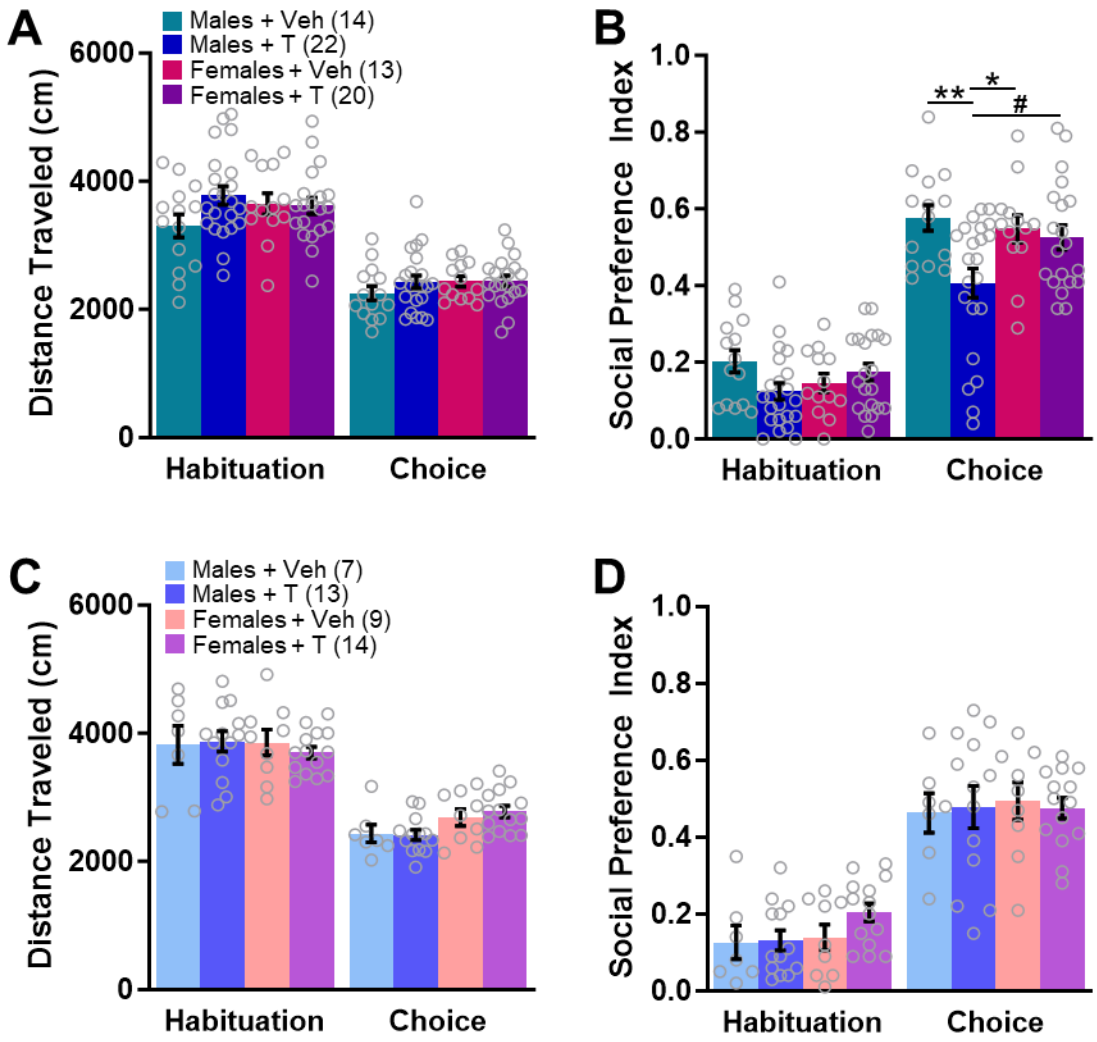

Figure 3. Testosterone administration on the day of birth but not on PN 18 induces social approach deficits in adolescent males. (A) During a 10 min habituation period of the social approach test, all experimental groups traveled similar distances. There were no group differences in distance traveled during the choice phase, in which a novel object and novel social partner mouse were present. (B) Males treated neonatally with testosterone had a significantly lower social preference index than males or females treated with vehicle, and neared significance compared to females treated with testosterone ($p=0.058$). (C) Male and female adolescent mice exhibited no significant differences in distance traveled regardless of treatment with veh or T on PN 18. (D) Treatment with T on PN 18 did not affect social preference index in males or females compared to veh controls. *$p<0.05$, **$p<0.01$, #$p<0.10$.

Figure 4

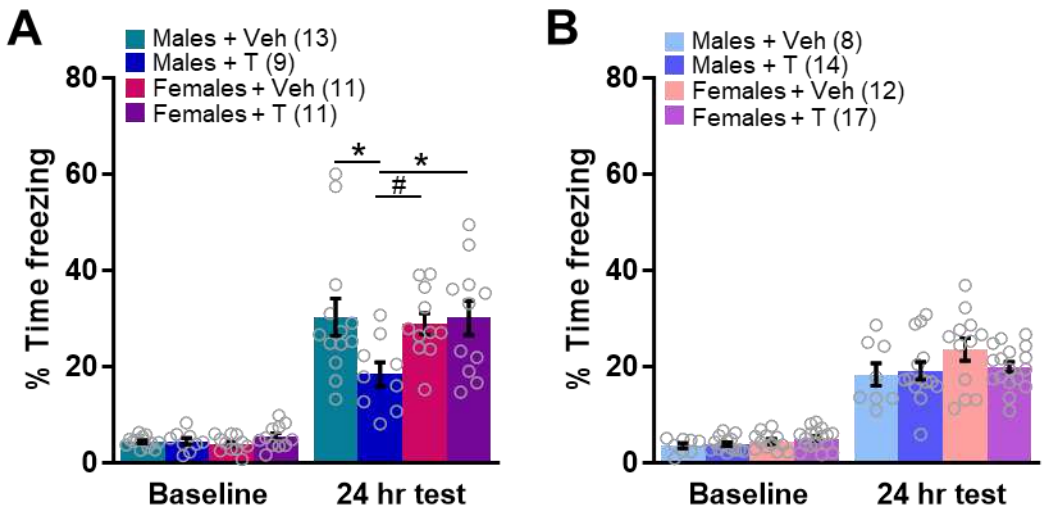

Figure 4. Testosterone administration on the day of birth but not on PN 18 induces contextual fear conditioning deficits in adult males. (A) Adult males treated on the day of birth exhibited significantly less freezing during a 24 hr test than those treated with veh or females treated with T, and trended toward significance compared to females treated with vehicle (p=0.066). (B) Treatment with T on PN 18 did not affect percent of time spent freezing in a 24 hr test in males or female adults compared to those treated neonatally with veh. *p<0.05, **p<0.01, #p<0.10.