Pubertal Androgens Reduce the Effects of Social Stress on Anxiety-related Behaviors in California Mice

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Abstract

Adolescence is an important developmental period during which anxiety-related behaviors differentiate in males and females. In humans anxiety prevalence increases to a greater degree in women than men after puberty, but the mechanism is unknown. We used social defeat stress to model anxiety behaviors in California mouse, a species in which aggressive females allow for comparison of social anxiety behaviors across sex. Adult female California mice show reduced social approach and increased social vigilance after exposure to stress, while these changes are weaker in males. Here we show that in juveniles, social defeat reduces social approach and increases social vigilance in both males and females. Next, we show that prepubertal castration sensitizes adult males to social defeat. However, when pubertal castration was paired with either testosterone or dihydrostesterone replacement, effects of defeat on social approach and vigilance were blunted in adult males. We also showed that effects of defeat on social behavior in juveniles were oxytocin receptor dependent, as has been described for adult females. This work highlights the importance of pubertal testosterone to the development of sex differences in anxiety behavior, and provides evidence that androgen receptors play an important role in the development of neural circuits of anxiety.

Introduction

Anxiety disorders are the most commonly diagnosed group of mental illness, with up to 20 percent of adults experiencing one within their lifetime [1–3]. However, despite the prevalence of anxiety disorders, current treatments are limited and not beneficial to all patients. Benzodiazepines are the most widely prescribed anti-anxiety medication and while they tend to have positive short-term effects, less than two thirds of patients will experience remission [4]. Selective serotonin reuptake inhibitors (SSRIs) are also widely used for anti-anxiety purposes but many do not respond to this treatment either [5]. Identification of underlying mechanisms involved with anxiety could contribute to novel approaches for treatments. Epidemiological studies report that women are twice as likely to be diagnosed with an anxiety disorder than men [1–3,6–10]. The basis of this disparity is unknown. One clue is the lack of sex differences in rates of childhood anxiety [3,6], with the prevalence of anxiety disorders in women increasing only at onset of puberty [3,6,11,12]. This suggests there may be sex-dependent pubertal maturation of neural circuits of anxiety. Supporting this hypothesis, work in rodents indicate that gonadal hormones act during puberty to shape behaviors such as anxiety [13] and learning strategies [14] in adulthood.

Psychosocial stress is a well-known risk factor for developing anxiety disorders [15–17]. Social defeat stress is a model of chronic social stress in rodents [18,19] that allows for the study of neural circuits of stress-related behavioral phenotypes. Most of this work has focused on males, with only a few models of female defeat stress [20–22]. The California mouse (*Peromyscus californicus*) model allows for the inclusion of both males and females while paying heed to ethological experiment design. This species has a monogamous social structure, where both

members of a pair jointly defend a shared territory. Thus both males and females exhibit aggressive behavior. Adult female California mice exposed to social defeat stress show robust and consistent decreases in social approach in a social interaction test. Adult females also show increased "vigilance", defined as time the focal mouse spends withdrawn from but orienting towards a novel stimulus mouse. When seen in human children, the combination of social withdrawal and vigilance is called behavioral inhibition and is a risk factor for the later development of anxiety disorders [15,17]. Unlike in adult females, adult male California mice do not show social withdrawal or increased vigilance behavior after experiencing social defeat. Males are affected by defeat stress as evidenced by increased anxiety behaviors and sucrose anhedonia that are present after defeat [23], decreased aggression [24], and impaired cognitive flexibility [25]. Curiously, adult gonadectomy has no impact on sex differences in social approach following defeat [26], nor does estrous cycle affect vulnerability to defeat in social approach [27]. This strongly implicates that sex differences in social-anxiety behaviors are programmed at some earlier point during development.

The short duration of juvenile/adolescent developmental periods in most rodents makes it difficult to conduct lengthy studies that remain contained within a particular developmental stage. Here we use the California mouse model to determine whether sex differences in stress-induced social anxiety behavior emerge during puberty. First, we delineate the timeline of pubertal maturation in California mice. We found that California mice experience a later onset of puberty and a slower rate of pubertal development than seen in mice/rats, only reaching adult maturity at about 90 days of age. This allows us to examine sustained changes in anxiety behaviors after exposure to social defeat stress, all before the onset of puberty. Next, we show

that sex differences in stress-induced social anxiety behavior are absent in pre-pubertal California mice. We further confirm that expression of anxiety behaviors is oxytocin receptor dependent in both juvenile males and females. We then use prepubertal castration to show that males deprived of typical gonadal hormone exposure do not develop an adult male typical social anxiety response. Next, we tested whether testosterone replacement during puberty was sufficient to induce the typical reduced anxiety response of adult males following social defeat. Here we found that pubertal testosterone is necessary to masculinize California mice's response to social defeat stress. We further find that administration of the non-aromatizable androgen dihydrotestosterone (DHT) has the same effect, indicating an important role of androgen receptors. Overall these results show that pubertal testosterone plays an important organizational role in the masculinization of male social anxiety behavior.

Methods

Animals

All studies were conducted with California mice (*Peromyscus californicus*) raised in a colony at UC Davis. Mice were housed in same sex groups (2-4) in clear polypropylene cages with Sani-Chip bedding (Harlan Laboratories, Indianapolis, IN, USA), Nestlets (Ancare, Bellmore, NY, USA) and Enviro-Dri (Eco-bedding, Fibercore, Cleveland, OH, USA). Mice were kept on a 16L:8D light cycle and had ad libitum access to food and water. All mouse care and experimental proceedings were in approval with the Institutional Animal Care and Use Committee at the University of California, Davis and in accordance with National Institutes of Health (NIH) guidelines.

Puberty Quantification

For measures of vaginal opening, preputial separation, weight, and coat color mice were briefly anesthetized (>1 minute isoflurane) before being weighed, photographed, and tested for vaginal opening/preputial separation. For gonad to body ratio, mice were weighed, briefly anesthetized (>1 minute isoflurane), euthanized, and then the uterus or testes were dissected out and weighed. Trunk blood was collected at time of euthanasia and plasma was extracted before being stored at -80C. Retro orbital blood samples were also collected. Here mice were briefly anesthetized (>1 minute isoflurane) before retro orbital samples were collected, then blood plasma was extracted and stored at -80C. Together the trunk blood and retro orbital plasma samples were used to determine plasma concentrations of testosterone and progesterone. Plasma was extracted and hormones were on a QTRAP 550 quadruple linear ion trap mass spectrometer equipped with an atmospheric pressure chemical ionization source (LC-MS/MS) as previously described [28]. Quantitative results were recorded as multiple reaction monitoring (MRM) area counts after determination for the response factor for each hormone and internal standard.

Juvenile California mice have a dark pelage and undergo a coat molt during adolescence to a golden brown adult pelage [29]. To quantify the percentage of adolescent coat molt we used methods derived from work in mice [30]. Photographs were modified using image editing software GIMP. Firstly the background was colored red (R = 254, G = 0, B = 0), secondly the magic wand tool was used to select all juvenile coat coloration which was then colored brown (R= 255, G=255, B=0), thirdly the magic wand tool was used to select all adult coat coloration

which was then colored yellow (R= 96, G=57, B=18). It is important to note that with this method a mouse with even a fully molted adult type coat will still register some small amount of juvenile (i.e. brown) coloration. The images where then exported in the statistical software R, which was then used to quantify the ratio of brown to yellow pigment in the image. The resulting data was then used to model the progress of the adolescent coat molt across age.

Experiment 1: Juvenile Social Defeat

Male and female juveniles (P34-36) underwent 3 consecutive days of social defeat stress (or control handling), with protocol as described previously [27]. During each episode of defeat the test mouse was placed in the home cage of a novel male-female adult resident pair. The opposite sex resident mouse was removed from the cage prior to the start of the defeat session. Each session of defeat lasted for 7 minutes, or until the test mouse received 7 bites. Control mice were placed into a clean, empty cage for 7 minutes across 3 consecutive days. Immediately before the first and third days of defeat/control, mice were transferred to the testing room and placed in an empty cage for 5 minutes. Behavior was filmed and quantified for a conditioned grooming response found in stressed mice [31].

On P50 mice underwent a social interaction test. The social interaction test was composed of 3 parts: an open field phase, an acclimation phase, and an interaction phase. In the open field phase the mouse was placed into an open area and allowed to explore it for 3 minutes. In the acclimation phase, an empty cage was placed in one end of the arena and the mouse allowed to explore for 3 minutes. In the interaction phase, a caged, unknown adult conspecific replaced the empty cage at one end of the arena, and the mouse was allowed to

explore for 3 minutes. Time in the interaction zone (a radius of 8 cm from the cage) (which we term as "social approach"), distance traveled, and time spent in corners was scored with AnyMaze software [26]. Duration of vigilance behavior was hand scored from a video recording. Vigilance was defined as any time the test mouse was sitting still, head oriented toward the target mouse, while outside of the interaction zone [32].

Experiment 2: Juvenile Social Defeat + OTA Injections

All male and female juveniles post-natal day 34-36 (P34-36) underwent 3 days of social defeat stress. All mice experienced defeat stress. At P50 mice were tested in a social interaction test. 30 minutes before the start of the social interaction test, each mouse received an intraperitoneal (i.p.) injection of 5mg/kg or 10mg/kg of the oxytocin antagonist (OTA) L-368,899 [32], or an injection of vehicle control (sterile phosphate buffered saline (PBS)).

Experiment 3: Prepubertal Castration

Male juveniles were castrated or underwent sham surgery between P35 and P40 [33]. During castration mice were exposed to 2-3% isoflurane for the duration of the surgery (approx. 30 minutes). A one-time injection of 0.1mg/kg buprenorphine was administered on the day of surgery. A 5mg/kg injection of carprofen was given on the day of surgery and the 2 days immediately following surgery. A group of non-surgery controls was included to control for possible effects of early life exposure to the anesthesia isoflurane [34]. These mice received no manipulations until the start of social defeat stress/control handling. At P90-92 all mice

underwent 3 days of either social defeat or control handling. At P106 mice where tested in a social interaction test.

Experiment 4: Prepubertal Castration with Hormone Replacement

Male juveniles were castrated between P35 and P40. At the time of surgery, each mouse received a subcutaneous implant. Implants were made out of silastic tubing (i.d. 0.04 in, o.d. 0.085 in) and contained either 1mm testosterone or 1mm dihydrotestosterone and were sealed with Dowsil Sealant (3145 RTV MIL-A-46146). Control implants were filled entirely with sealant. Previous work showed that this sized T yields a plasma level of T of 0.81 ± 0.05 ng/ml [35] which is comparable to mean T levels of intact male California mice [36]. At P90-92 all mice underwent 3 days social defeat. At P106 mice were tested in a social interaction test.

Statistics

All statistical analyses were performed using R statistical software. Normality was assessed via QQPlot. A Flinger-Killen test was used to assess homogeneity of variance. If data met the necessary assumptions, a two-way ANOVA , or one-way ANOVA (Experiment 4) was used to test for significant main effects. For an ANOVA that showed a significant main effect, planned pairwise comparisons were used to determine differences between control and manipulated groups. If data did not meet the assumptions necessary to conduct an ANOVA, a Kruskal-Wallis test was used instead. Kruskal-Wallis was used for analysis of vigilance data in Experiment 1 (juveniles only) and Experiment 3. In the case of a significant Kruskal-Wallis test, a Mann-Whitney *U* test was used to determine differences between groups. Simple linear regressions

were used when modeling progress of the adolescent coat molt in males and females over time.

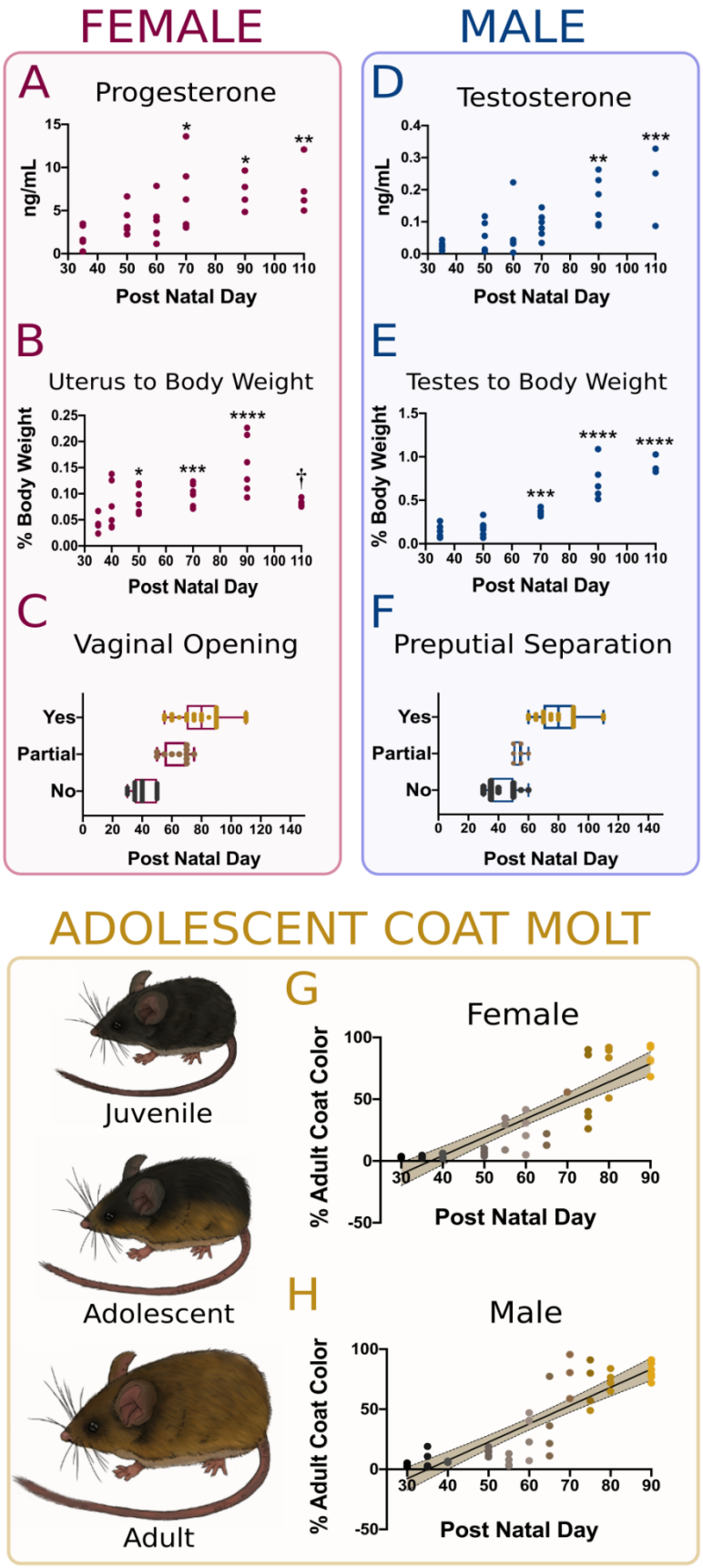
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Fig. 1. Measures of pubertal maturation across adolescence in the California mouse. A. Blood plasma progesterone concentration in females. **B.** Percentage of uterus to body weight in females. **C.** Measure of vaginal opening in females. **D.** Blood plasma testosterone concentration in males. **E.** Percentage of testes to body weight in males. **F.** Measure of preputial separation in males. **G/H.** Percent adult pelage coloring across adolescence in females and males. n=3-5 per post-natal day for all analysis. † p=0.07, * p<0.05, ** p<0.001, *** p<0.0001, **** p<0.00001

Results

Puberty Quantification

We quantified measures of puberty on male and female mice across multiple age groups (Fig 1). We found a significant effect of age on plasma testosterone levels in males ($F_{5,28}$=4.77, p=0.0028), with males at P90 (p=0.004) and P110 (p=0.0003) having significantly more plasma testosterone than P35 juveniles (Fig 1D). We found a trending effect of age on plasma progesterone levels in females ($F_{5.26}$=2.56, p=0.05), with females at P70 (p=0.01), P90 (p=0.01), and P110 (p=0.005) having significantly more plasma progesterone than P35 juveniles (Fig 1A). There was a significant effect of age on percentage of testes to body weight in males ($F_{1,21}$=112.3, p=6.96x10^{-10}), with males at P70 (p=0.00007), P90 (p=1.5x10^{-6}), and P110 (p=2.6x10^{-7}) having a significantly larger testes to body percent (Fig 1E). There was a significant effect of age on percentage of uterus to body weight in females ($F_{1,32}$=6.99, p=0.01), with females at P50 (p=0.02), P70 (p=0.01), and P90 (p=6.7x10^{-6}) having significantly larger uterus to body percent than P35 juveniles (Fig 1B). There was a trend for females at P110 (p=0.07) to have larger uterus to body percent compared to P35 juveniles (Fig 1B). We found age to have a positive correlation with stage of adolescent coat molt in males (R^2=0.75, $F_{1,44}$=131.6, p<0.0001) (Fig 1H) and females (R^2=0.75, $F_{1,41}$=125.3, p<0.0001) (Fig 1G).

Experiment 1

Juvenile males and females underwent social defeat stress and were tested in a social interaction test 2 weeks later (Fig 2). Exposure to social defeat stress reduced the amount of time spent in the interaction zone during the interaction phase of the test ($F_{1,27}$=47.09, p=2.3x10^{-7}) in both males (p=0.0001) and females (p<0.00001) (Fig 2B). During the acclimation phase there was a significant effect of sex on time in the interaction zone ($F_{1,27}$=6.64, p=0.016) with females spending more time with the empty cage than males (p=0.01). There was no effect of stress. During the interaction phase, defeated juveniles also showed significantly increased vigilance behavior (Kruskal-Wallis, $\chi^2(3)$=16.33, p=0.0009) in males (p=0.03) and females (p=0.02) (Fig 2C). There were no differences seen in vigilance behavior during the acclimation phase. There were also no difference in distance traveled or time spent in the arena's corners during the open field phase of the test.

To compare these results in juveniles with adults, we performed an analysis of adult data from published and unpublished studies spanning from 2010-2020 to determine measures of social approach in adults (Fig 2). There was a significant interaction of sex and stress on time in the interaction zone ($F_{1,625}$=13.36, p<0.0003), with stressed females showing significantly less social approach than control females (p=2.0x10^{-16}) (Fig 2D). We did the same for measures of vigilance working with data from published and unpublished studies spanning from 2018-2020. There was a significant interaction of sex and stress on the duration of vigilance ($F_{1,164}$=8.44, p=0.004), with stressed females showing significantly more vigilance than control females (p=2x10^{-16}) (Fig 2E). We also found that stressed males showed significantly more vigilance than control males (p=0.0003) and there was a significant difference for stressed males to exhibit

less vigilance than stressed females (p=0.002) (Fig 2E). These analyses are consistent with previous work showing the effects of social stress on vigilance are stronger in females than males [37].

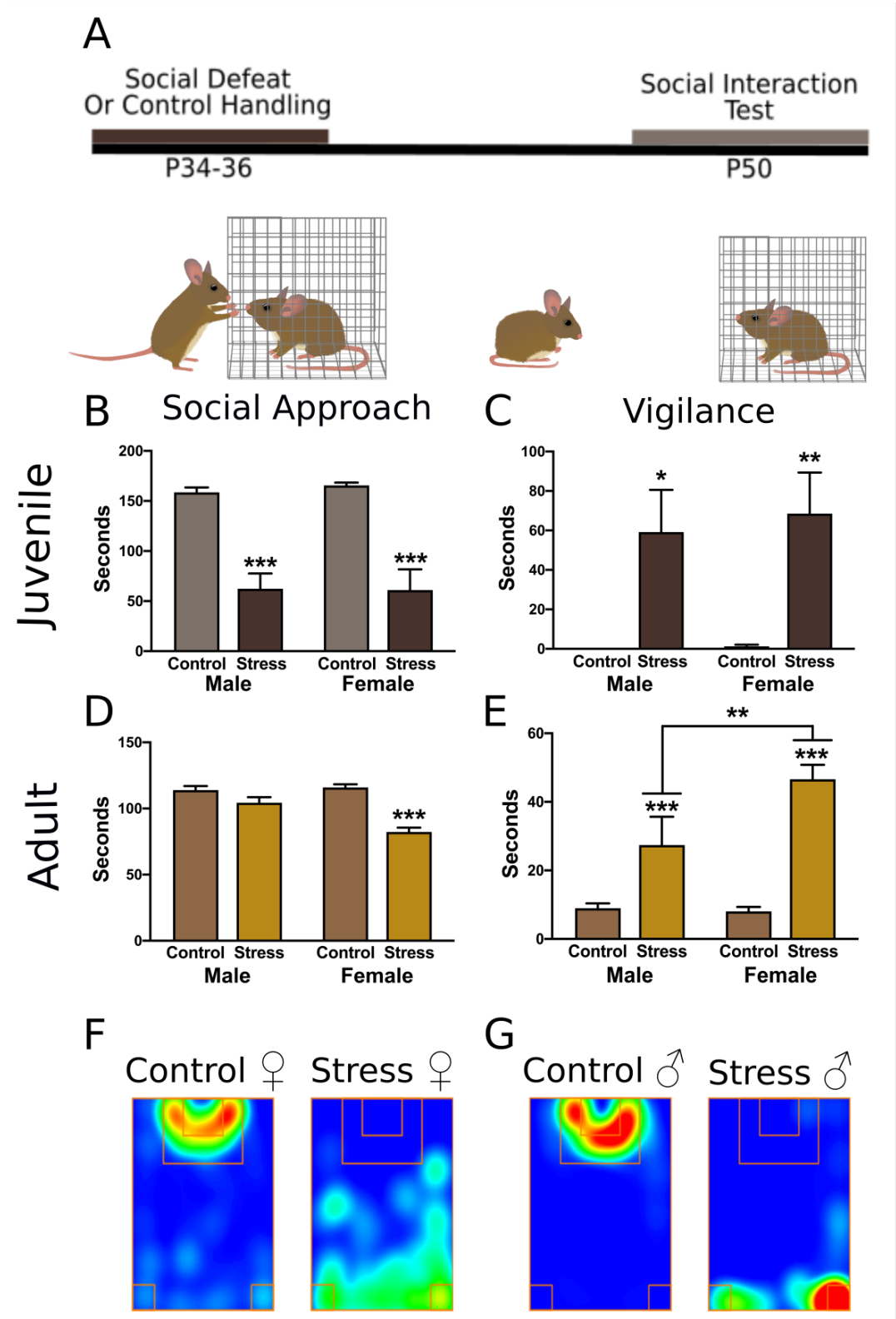

Fig. 2. Social defeat stress induces high levels of social anxiety behavior in juvenile males and females. A. Timeline of Experiment 1. **B.** Stressed juvenile males and females show decreased rates of social approach (n=6-9/group). **C.** Stressed juvenile males and females show increased levels of vigilance (n=6-9/group). **D.** Stressed adult females show decreased rates of social approach (n=84-203/group). **E.** Stressed adult males show increased levels of vigilance, while stressed adult females show increased levels of vigilance both versus control and when compared to stressed adult males (n=10-61). **F/G.** Representative heatmaps for the interaction phase showing reduced social approach in stressed juvenile males and females compared to control. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$

Experiment 2

Juvenile males and females underwent social defeat stress and were tested in a social interaction test 2 weeks later (Fig 3). 30 minutes before the start of social defeat mice received an intraperitoneal (i.p.) injection of either 5mg/kg, 10mg/kg OTA, or vehicle control. We found no differences in distance traveled or time spent in corners during the open field phase of the test. During the acclimation phase of the test, there were no differences when examining time in the interaction zone or vigilance. During the interaction phase of the test, there was a significant effect of OTA treatment on time in the interaction zone ($F_{2,42}=8.42$, $p=0.0008$), with both 5mg/kg treated males ($p=0.01$) and females ($p=0.01$) showing significantly more social approach than vehicle treated mice (Fig 3B). There was also a significant effect of OTA treatment on vigilance ($F_{2.39}=7.99$, $p=0.001$) with 5mg/kg treated males ($p=0.04$) and females ($p=0.004$) showing significantly less vigilance than vehicle treated mice (Fig 3C).

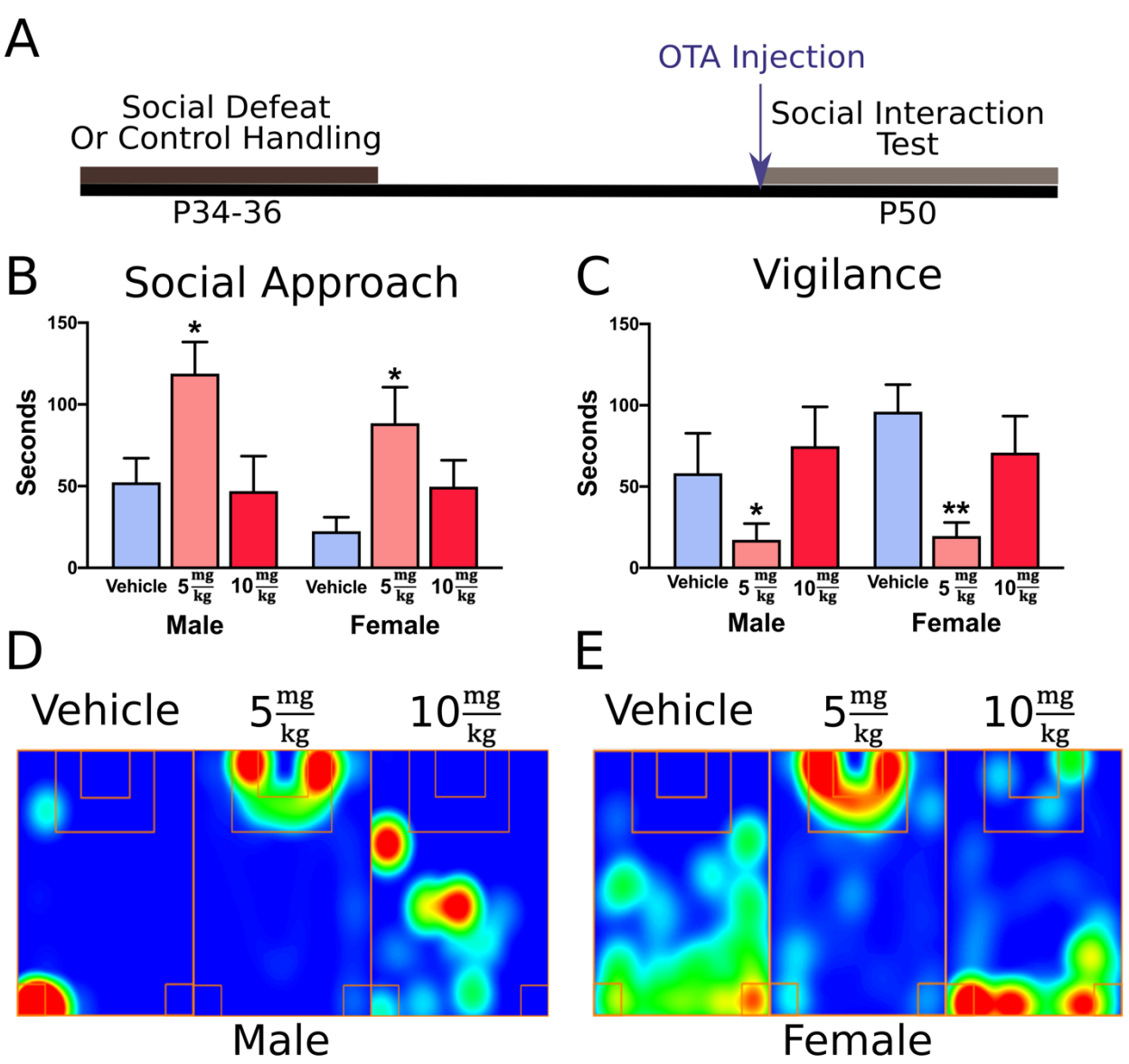

Fig 3. OTA injection ablates social anxiety behavior in stressed juvenile males and females. A. Timeline of Experiment 2. **B.** A 5mg/kg i.p. injection of OTA rescues social approach in stressed male and female juveniles (n=7-8/group). **C.** A 5mg/kg i.p. injection of OTA reduces vigilance in stressed male and female juveniles (n=7-8/group).). **D/E.** Representative heatmaps for the interaction phase showing reduced social approach stressed juvenile males and females is rescued by a 5mg/kg injection of OTA. * $p<0.05$, ** $p<0.001$

Experiment 3

Juvenile males were either castrated prepubertally, received a sham castration, or received no surgery at all (Fig 4). Mice were allowed to grow to adulthood, before receiving either 3 days of social defeat stress or control handling. 2 weeks later mice were tested in a social interaction test. During the open field phase of the test we found no differences in time spent in corners or distance traveled. During the acclimation phase of the test we found no differences in time spent in the interaction zone or vigilance. During the interaction phase of the test we found significant effects of exposure to social defeat ($F_{1,39}$=1694, p=0.0002), castration ($F_{2,39}$=4.34, p=0.02), and a trend for an interaction between the two (Fig 4B, $F_{2,39}$=3.2, p=0.05) for social approach. In castrated males, stress reduced social approach (p<0.0001) but there were no effects of stress in sham or no surgery controls (Fig 4B). There was also a significant interaction between social defeat stress and castration on vigilance behavior (Kruskal-Wallis, $\chi2(5)$=17.51, p=0.0036), with stress increasing vigilance in castrated mice (Fig. 4C, p<0.004) but not in sham castration or no surgery controls.

Experiment 4

Juvenile males were prepubertally castrated and then received one of 3 types of subdermal implants: control, testosterone, or dihydrotestosterone (Fig 4). Mice were allowed to grow to adulthood before receiving 3 days of social defeat stress. 2 weeks later mice were tested in a social interaction test. During the open field phase of the test we found no differences in time spent in the corners of distance traveled. During the acclimation phase we found no differences in time spent in the interaction zone or vigilance. During the interaction phase we found a significant effect of hormone implant on time in the interaction zone ($F_{2,11}$=7.73, p=0.008), with

testosterone (p=0.0026) and DHT (p=0.033) treated males showing increased time in interaction zone compared to control (Fig 4H). We also found a significant effect of hormone implant on vigilance ($F_{2,11}$=12.17, p=0.0016), with testosterone (p=0.0008) and DHT (p=0.003) treated males showing decreased vigilance compared to control (Fig 4I).

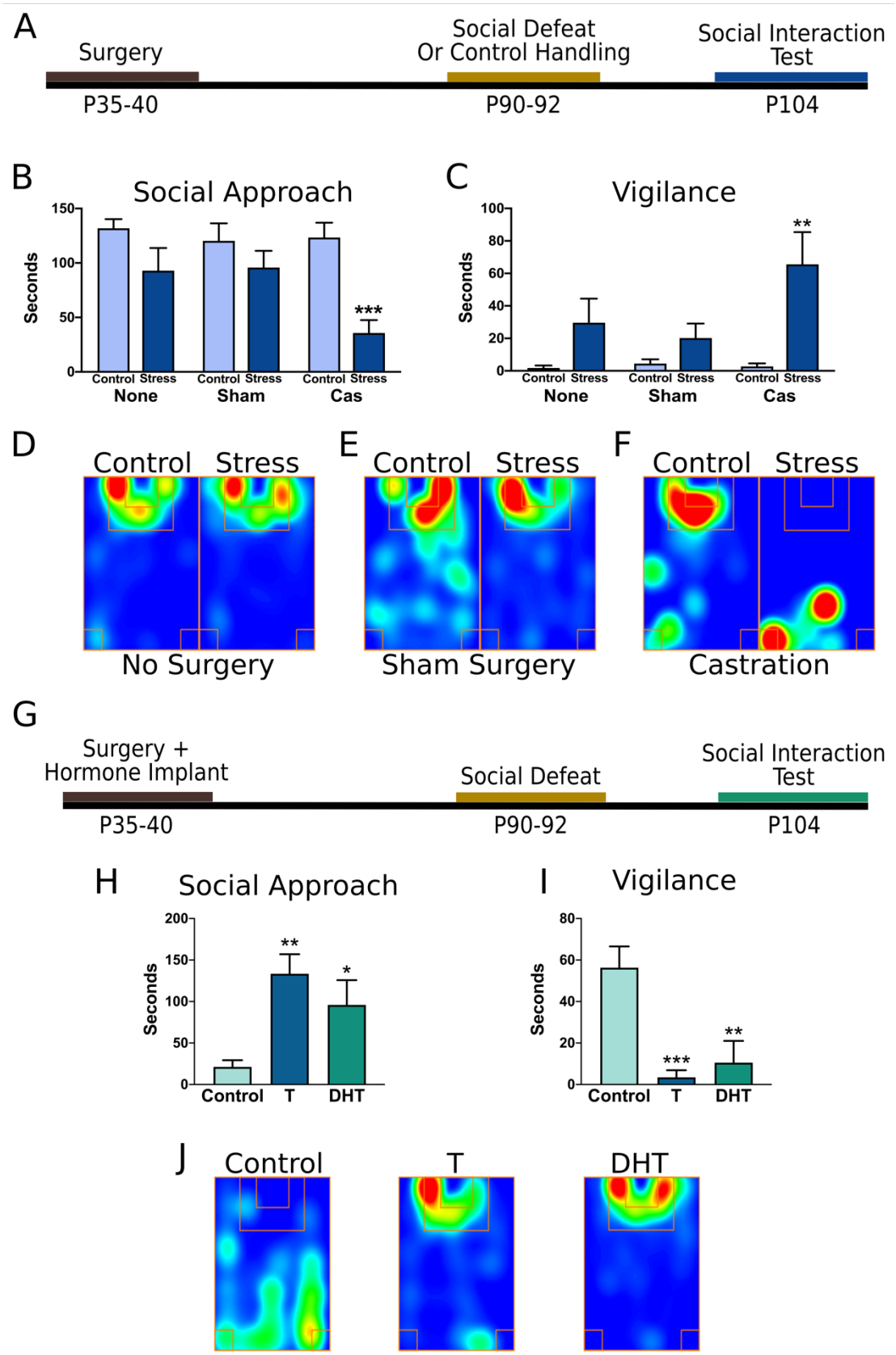
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Fig. 4. Prepubertal castration increases social anxiety behavior in stressed adult males, while testosterone or dihydrotestosterone implant rescues. A. Timeline of Experiment 3. **B.** Prepubertally castrated males show decreased social approach after social defeat stress (n=7-8/group). **C.** Prepubertally castrated males show increased vigilance after social defeat stress (n=7-8/group). **D/E/F.** Representative heatmaps for the interaction phase showing reduced social approach in stressed prepubertally castrated males. **G.** Timeline of Experiment 4. **H.** Testosterone or dihydrotestosterone implant rescues social approach in prepubertally castrated males (n=4-5/group). **I.** Testosterone or dihydrotestosterone implant ablates vigilance in prepubertally castrated males (n=4-5/group). **J.** Representative heatmaps for the interaction phase showing rescued social approach in testosterone and dihydrotestosterone implant males. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$

Discussion

A decade of studies using the California mouse model of social defeat has produced consistent sex differences in the social interaction test, with adult females exhibiting more social-anxiety related behaviors than males. However, the basis of this sex difference has remained unknown, since these sex differences are sustained even when gonads are removed [26]. Here we demonstrate that sex differences in social anxiety behavior are absent in prepubertal California mice, mirroring patterns seen in humans [6,38]. In both male and female juveniles, social defeat decreased social approach and increased vigilance behavior, which is dependent on the activation of oxytocin receptor, as has been described for adult female California mice [32]. Prepubertal castration prevents the development of sex differences in stress-induced social anxiety, and exposure to testosterone during pubertal development is sufficient to restore this sex difference. The finding that DHT replacement has similar effects suggests that activation of androgen receptors during puberty is a key mechanism underlying this effect. These results highlight the importance of adolescent developmental both on the emergence of sex differences in adult behavior and in the maturation of neural circuits of anxiety.

Androgens have an important role in the masculinization of the male brain. Starting from before birth testosterone works to shape the development of neural circuits. The perinatal time period is seen as a critical period when sex hormones work to masculinize and defeminize the male brain [39,40]. Puberty is a second critical period wherein testosterone has profound impacts on the shape of neural development [41–44]. Gonadal hormone levels increase substantially across the course of puberty, with male testosterone rising to adult typical expression in later puberty [45]. Prepubertal castration in rodents has been previously shown to alter adult behavior in a various number of ways. The best known examples are decreased expression of male sexual behavior [46] and decreased intrasexual aggression [47]. Indeed, the absence of gonadal hormones during puberty impairs male sexual behavior even if testosterone capsules are provided in the adult [48]. Similar results are seen with intrasexual aggression [49]. In contrast, mice that are intact during puberty continue to show high levels of intrasexual aggression even after adult castration [49]. These data show the importance of normal levels of testosterone exposure during the pubertal critical period in shaping brain development.

Testosterone exposure also has important impacts on behavioral and endocrine stress responses. Studies in adult male mice have found that testosterone injections can have anxiolytic effects in elevated plus maze [50] and open field [51]. Testosterone has also been shown to decrease plasma levels of ACTH in response to a restraint stress [52]. However, testosterone does not elicit anxiolytic effects in males of all developmental stages. In fact, testosterone administration in prepubertal males has been shown to have no effect on ACTH response to restraint stress [53]. Interestingly, one study showed prepubertally castrated males show decreased measures of anxiety during adolescence and then increased anxiety behavior as

adults in an open field and elevated plus tests [54]. However other work has found prepubertal castration to have anxiogenic effects on adolescent behavior [13]. It is important to note that all examples of anxiety behaviors thus far discussed have been in non-social contexts. The fast maturation of commonly studied rodents (i.e. laboratory strains of mice and rats) make it difficult to study effects of chronic social stress during the juvenile period. There have been studies examining later impacts of social defeat stress in juvenile mice in adolescents [55], and adults [56], or examining acute effects of a social defeat episode in juveniles [56], but none examining chronic effects of social defeat stress completely within the juvenile or adolescent developmental period. As testosterone can exert neuromodulatory effects through the activity of either androgen or estrogen receptors, it is important to carefully and mechanistically consider their pubertal impact.

The results of Experiments 3 and 4 showcase the importance of pubertal testosterone to the masculinization of social anxiety behavior. However, it is important to acknowledge the testosterone and DHT implants given in the prepubertal castration and hormone replacement study (Experiment 4) were based on the average level of testosterone in a sexually naïve adult male [35,57]. Thus, mice experienced elevated androgen levels at an earlier age than a typically developing male California mouse. However, testosterone and DHT treatments abolished the social anxiety response observed in prepubertally castrated males, suggesting that these treatments mimicked the normal development of intact males. Historically sexual differentiation of the brain has focused on the roles of testosterone aromatized into estradiol [58], while androgen receptors have been considered more important for masculinization of the peripheral nervous system and spinal cord [59]. Since we found that non-aromatizable androgen

(DHT) impacts behavior, we infer an important role of androgen receptor on the masculinization of neural circuits of social anxiety. This suggests that androgen receptors may have an underappreciated impact on brain development during puberty. Prepubertal castration decreases the prevalence of long term potentiation (LTP) in the hippocampus of male rats [60–62], and work with DHT suggests this to be mediated through binding to androgen receptor [60]. Interestingly, prepubertally castrated males have been shown to have both better social memory and reduced hippocampal LTP, while neither can be said for typically developing rats who later receive androgen receptor antagonists in adulthood [61]. This may indicate that there may be a sensitive period for testosterone influence on pathways of LTP formation during puberty. Social stress has been shown to increase LTP in the extended amygdala [63]. Particularly within the BNST, studies in California mice have found social defeat stress induced increases in BDNF (which helps trigger the formation of LTP [64]) in adult females but not males [65]. We know the BNST plays a main role in the presentation of social anxiety behavior, which is driven by the activity of BNST oxytocin neurons. An infusion of oxytocin into the BNST of unstressed, adult male or female California mice is sufficient to induce social anxiety behaviors in both sexes [37], and a systemic injection of OTA will attenuate social anxiety behaviors in stressed adult females [32]. Here we found the same was true in stressed male and female juveniles, with a 5mg/kg systemic OTA injection increasing social approach and reducing vigilance. Interestingly, we observed an inverted U shaped dose-response curve of OTA administration with the 10mg/kg OTA injection not ablating social anxiety behaviors. We hypothesize this may be due to the systemic nature of the injection causing suppression of oxytocin pathways that promote social approach [66] at the higher dose. Together, these results suggest testosterone acting via

androgen receptors during puberty play a key role in the sexual differentiation of oxytocin-dependent circuits of social anxiety.

California mice are long-lived, and the slower development of this species makes it particularly suited to the study of adolescence development. Here we show that female California mice do not begin showing vaginal opening until after post-natal day 50, in contrast to laboratory mice (*Mus musculus*) who typically experience vaginal opening around post-natal day 30 [67,68]. Our findings also place vaginal opening to be about a week later than an earlier study in California mice [69]. In male California mice preputial separation was not noted until after an average of post-natal day 60, while in *Mus musculus* it occurs by an average of post-natal day 44 [70]. Male California mice only show significantly increased levels of testosterone hormones by post-natal day 90, while *Mus musculus* reach there by post-natal day 40 [45]. Even then, markers of full adult sexual maturity in the California mouse (i.e. adolescent coat molting) can continue past post-natal day 90. Overall *Mus musculus* are considered to have reached adult maturity by about post-natal day 56 [68], while California mice are shown to take until about post-natal day 90. This leaves a much greater time span within which to conduct experiments both in the juvenile and adolescent developmental periods, and we posit that the California mouse is an ideal model to explore mechanisms within those time periods.

When considering global trends of anxiety disorders in humans, it becomes clear there is a prominent sex difference with women showing higher rates of diagnosis than men. This is not a consistent difference across the lifespan, but rather one that emerges with adolescence. However, the current literature cannot explain the origin of this sex difference. Here we used the California mouse model to examine the role of testosterone in the masculinization of adult

male response to social stress. We found that pubertal testosterone is necessary to induce these changes and that activation of androgen receptors is sufficient to do so. This provides a powerful basis for understanding the mechanisms behind sex differences in anxiety behavior and the neural mechanisms that drive it. Future directions should examine effects of androgens on brain regions regulating social anxiety behavior, such as the bed nucleus stria terminalis or prefrontal cortex. It would also be valuable to examine if pubertal testosterone has similar effects on response to different modes of stressors, or if this is a purely social stress phenomenon.

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References

1. Hollingworth, S. A., Burgess, P. M. & Whiteford, H. A. Affective and anxiety disorders: prevalence, treatment and antidepressant medication use. *Aust. N. Z. J. Psychiatry* **44**, 513–519 (2010).
2. Remes, O., Brayne, C., Linde, R. van der & Lafortune, L. A systematic review of reviews on the prevalence of anxiety disorders in adult populations. *Brain Behav.* **6**, e00497 (2016).
3. Wesselhoeft, R., Pedersen, C. B., Mortensen, P. B., Mors, O. & Bilenberg, N. Gender–age interaction in incidence rates of childhood emotional disorders. *Psychol. Med.* **45**, 829–839 (2015).
4. Cassano, G. B., Rossi, N. B. & Pini, S. Psychopharmacology of anxiety disorders. *Dialogues Clin. Neurosci.* **4**, 271–285 (2002).
5. Baldwin, D., Woods, R., Lawson, R. & Taylor, D. Efficacy of drug treatments for generalised anxiety disorder: systematic review and meta-analysis. *BMJ* **342**, d1199 (2011).
6. Beesdo, K. *et al.* Incidence of social anxiety disorder and the consistent risk for secondary depression in the first three decades of life. *Arch. Gen. Psychiatry* **64**, 903–912 (2007).
7. Hunt, C., Issakidis, C. & Andrews, G. DSM-IV generalized anxiety disorder in the Australian National Survey of Mental Health and Well-Being. *Psychol. Med.* **32**, 649–659 (2002).
8. Husain, N., Creed, F. & Tomenson, B. Depression and social stress in Pakistan. *Psychol. Med.* **30**, 395–402 (2000).

9. Ma, X. *et al.* Generalized Anxiety Disorder in China: Prevalence, Sociodemographic Correlates, Comorbidity, and Suicide Attempts. *Perspect. Psychiatr. Care* **45**, 119–127 (2009).
10. Munk-Jørgensen, P. *et al.* Prevalence of Generalized Anxiety Disorder in General Practice in Denmark, Finland, Norway, and Sweden. *Psychiatr. Serv.* **57**, 1738–1744 (2006).
11. Dahl, R. E. Adolescent brain development: a period of vulnerabilities and opportunities. Keynote address. *Ann. N. Y. Acad. Sci.* **1021**, 1–22 (2004).
12. Davey, C. G., Yücel, M. & Allen, N. B. The emergence of depression in adolescence: development of the prefrontal cortex and the representation of reward. *Neurosci. Biobehav. Rev.* **32**, 1–19 (2008).
13. Boivin, J. R., Piekarski, D. J., Wahlberg, J. K. & Wilbrecht, L. Age, sex, and gonadal hormones differently influence anxiety- and depression-related behavior during puberty in mice. *Psychoneuroendocrinology* **85**, 78–87 (2017).
14. Delevich, K., Hall, C. D., Piekarski, D., Zhang, Y. & Wilbrecht, L. Prepubertal gonadectomy reveals sex differences in approach-avoidance behavior in adult mice. *Horm. Behav.* **118**, 104641 (2020).
15. Clauss, J. A. & Blackford, J. U. Behavioral Inhibition and Risk for Developing Social Anxiety Disorder: A Meta-Analytic Study. *J. Am. Acad. Child Adolesc. Psychiatry* **51**, 1066-1075.e1 (2012).
16. Kelly, M. M., Tyrka, A. R., Anderson, G. M., Price, L. H. & Carpenter, L. L. Sex differences in emotional and physiological responses to the Trier Social Stress Test. *J. Behav. Ther. Exp. Psychiatry* **39**, 87–98 (2008).
17. Silvers, J. A. *et al.* Vigilance, the Amygdala, and Anxiety in Youths With a History of Institutional Care. *Biol. Psychiatry Cogn. Neurosci. Neuroimaging* **2**, 493–501 (2017).
18. Golden, S. A., Covington, H. E., Berton, O. & Russo, S. J. A standardized protocol for repeated social defeat stress in mice. *Nat. Protoc.* **6**, 1183–1191 (2011).
19. Huhman, K. L. Social conflict models: can they inform us about human psychopathology? *Horm. Behav.* **50**, 640–646 (2006).
20. Harris, A. Z. *et al.* A Novel Method for Chronic Social Defeat Stress in Female Mice. *Neuropsychopharmacology* **43**, 1276–1283 (2018).
21. Iñiguez, S. D. *et al.* Vicarious Social Defeat Stress Induces Depression-Related Outcomes in Female Mice. *Biol. Psychiatry* **83**, 9–17 (2018).
22. Takahashi, A. *et al.* Establishment of a repeated social defeat stress model in female mice. *Sci. Rep.* **7**, 12838 (2017).
23. Williams, A. V. *et al.* Acute inhibition of kappa opioid receptors before stress blocks depression-like behaviors in California mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **86**, 166–174 (2018).
24. Steinman, M. Q. *et al.* Hypothalamic vasopressin systems are more sensitive to the long term effects of social defeat in males versus females. *Psychoneuroendocrinology* **51**, 122–34 (2015).
25. Laredo, S. A. *et al.* Effects of defeat stress on behavioral flexibility in males and females: modulation by the mu-opioid receptor. *Eur J Neurosci* **41**, 434–441 (2015).

26. Trainor, B. C. *et al.* Sex differences in stress-induced social withdrawal: independence from adult gonadal hormones and inhibition of female phenotype by corncob bedding. *Horm. Behav.* **63**, 543–550 (2013).
27. Trainor, B. C. *et al.* Sex Differences in Social Interaction Behavior Following Social Defeat Stress in the Monogamous California Mouse (<italic>Peromyscus californicus</italic>). *PLoS ONE* **6**, e17405 (2011).
28. Kraynak, M. *et al.* Extraovarian gonadotropin negative feedback revealed by aromatase inhibition in female marmoset monkeys. *Am. J. Physiol. - Endocrinol. Metab.* **313**, E507–E514 (2017).
29. Collins, H. H. Studies of the pelage phases and of the nature of color variations in mice of the genus Peromyscus. *J. Exp. Zool.* **38**, 45–107 (1923).
30. Ounpraseuth, S. *et al.* A Method to Quantify Mouse Coat-Color Proportions. *PLOS ONE* **4**, e5414 (2009).
31. Greenberg, G. D., Steinman, M. Q., Doig, I. E., Hao, R. & Trainor, B. C. Effects of social defeat on dopamine neurons in the ventral tegmental area in male and female California mice. *Eur J Neurosci* **42**, 3081–94 (2015).
32. Duque-Wilckens, N. *et al.* Oxytocin Receptors in the Anteromedial Bed Nucleus of the Stria Terminalis Promote Stress-Induced Social Avoidance in Female California Mice. *Biol Psychiatry* **83**, 203–213 (2018).
33. Trainor, B. C. & Marler, C. A. Testosterone, Paternal Behavior, and Aggression in the Monogamous California Mouse (Peromyscus californicus). *Horm. Behav.* **40**, 32–42 (2001).
34. Murphy, K. L. & Baxter, M. G. Long-Term Effects of Neonatal Single or Multiple Isoflurane Exposures on Spatial Memory in Rats. *Front. Neurol.* **4**, (2013).
35. Trainor, B. C., Bird, I. M. & Marler, C. A. Opposing hormonal mechanisms of aggression revealed through short-lived testosterone manipulations and multiple winning experiences. *Horm. Behav.* **45**, 115–121 (2004).
36. Trainor, B. C., Bird, I. M., Alday, N. A., Schlinger, B. A. & Marler, C. A. Variation in Aromatase Activity in the Medial Preoptic Area and Plasma Progesterone Is Associated with the Onset of Paternal Behavior. *Neuroendocrinology* **78**, 36–44 (2003).
37. Duque-Wilckens, N. *et al.* Extra-hypothalamic oxytocin neurons drive stress-induced social vigilance and avoidance. *bioRxiv* 2020.06.02.129981 (2020) doi:10.1101/2020.06.02.129981.
38. Wesselhoeft, R., Pedersen, C. B., Mortensen, P. B., Mors, O. & Bilenberg, N. Gender-age interaction in incidence rates of childhood emotional disorders. *Psychol. Med.* **45**, 829–839 (2015).
39. McCarthy, M. M. & Arnold, A. P. Reframing sexual differentiation of the brain. *Nat. Neurosci.* **14**, 677–683 (2011).
40. Wallen, K. & Baum, M. J. 69 - Masculinization and Defeminization in Altricial and Precocial Mammals: Comparative Aspects of Steroid Hormone Action. in *Hormones, Brain and Behavior* (eds. Pfaff, D. W., Arnold, A. P., Fahrbach, S. E., Etgen, A. M. & Rubin, R. T.) 385–423 (Academic Press, 2002). doi:10.1016/B978-012532104-4/50071-8.
41. Ahmed, E. I. *et al.* Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. *Nat. Neurosci.* **11**, 995–997 (2008).

42. Juraska, J. M., Sisk, C. L. & DonCarlos, L. L. Sexual differentiation of the adolescent rodent brain: Hormonal influences and developmental mechanisms. *Horm. Behav.* **64**, 203–210 (2013).
43. Schulz, K. M. & Sisk, C. L. Pubertal hormones, the adolescent brain, and the maturation of social behaviors: Lessons from the Syrian hamster. *Mol. Cell. Endocrinol.* **254–255**, 120–126 (2006).
44. Sisk, C. L. & Zehr, J. L. Pubertal hormones organize the adolescent brain and behavior. *Front. Neuroendocrinol.* **26**, 163–174 (2005).
45. Jean-Faucher, C., Berger, M., Turckheim, M. de, Veyssiere, G. & Jean, C. DEVELOPMENTAL PATTERNS OF PLASMA AND TESTICULAR TESTOSTERONE IN MICE FROM BIRTH TO ADULTHOOD. *Eur. J. Endocrinol.* **89**, 780–788 (1978).
46. Romeo, R. D., Richardson, H. N. & Sisk, C. L. Puberty and the maturation of the male brain and sexual behavior: recasting a behavioral potential. *Neurosci. Biobehav. Rev.* **26**, 381–391 (2002).
47. Barkley, M. S. & Goldman, B. D. The effects of castration and silastic implants of testosterone on intermale aggression in the mouse. *Horm. Behav.* **9**, 32–48 (1977).
48. Schulz, K. M. *et al.* Gonadal hormones masculinize and defeminize reproductive behaviors during puberty in the male Syrian hamster. *Horm. Behav.* **45**, 242–249 (2004).
49. McKinney, T. D. & Ingle, M. K. Intermale Aggression in Mice: Modification by Postneonatal Castration. *Proc. Soc. Exp. Biol. Med.* **145**, 340–342 (1974).
50. Aikey, J. L., Nyby, J. G., Anmuth, D. M. & James, P. J. Testosterone Rapidly Reduces Anxiety in Male House Mice (Mus musculus). *Horm. Behav.* **42**, 448–460 (2002).
51. Frye, C. A. & Seliga, A. M. Testosterone increases analgesia, anxiolysis, and cognitive performance of male rats. *Cogn. Affect. Behav. Neurosci.* **1**, 371–381 (2001).
52. Viau, V. Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. *J. Neuroendocrinol.* **14**, 506–513 (2002).
53. Romeo, R. D., Lee, S. J., Chhua, N., McPherson, C. R. & McEwen, B. S. Testosterone cannot activate an adult-like stress response in prepubertal male rats. *Neuroendocrinology* **79**, 125–132 (2004).
54. Renczés, E. *et al.* The age-dependent effect of pre-pubertal castration on anxiety-like behaviour in male rats. *Andrologia* **52**, e13649 (2020).
55. Iñiguez, S. D. *et al.* Social defeat stress induces a depression-like phenotype in adolescent male c57BL/6 mice. *Stress Amst. Neth.* **17**, 247–255 (2014).
56. Mouri, A. *et al.* Juvenile social defeat stress exposure persistently impairs social behaviors and neurogenesis. *Neuropharmacology* **133**, 23–37 (2018).
57. Trainor, B. C. & Marler, C. A. Testosterone promotes paternal behaviour in a monogamous mammal via conversion to oestrogen. *Proc. R. Soc. Lond. B Biol. Sci.* **269**, 823–829 (2002).
58. Schwarz, J. M. & McCarthy, M. M. Cellular mechanisms of estradiol-mediated masculinization of the brain. *J. Steroid Biochem. Mol. Biol.* **109**, 300–306 (2008).
59. Breedlove, S. M., Jacobson, C. D., Gorski, R. A. & Arnold, A. P. Masculinization of the female rat spinal cord following a single neonatal injection of testosterone propionate but not estradiol benzoate. *Brain Res.* **237**, 173–181 (1982).
60. Harley, C. W., Malsbury, C. W., Squires, A. & Brown, R. A. Testosterone decreases CA1 plasticity in vivo in gonadectomized male rats. *Hippocampus* **10**, 693–697 (2000).

61. Hebbard, P. C., King, R. R., Malsbury, C. W. & Harley, C. W. Two organizational effects of pubertal testosterone in male rats: transient social memory and a shift away from long-term potentiation following a tetanus in hippocampal CA1. *Exp. Neurol.* **182**, 470–475 (2003).
62. Moradpour, F., Fathollahi, Y., Naghdi, N., Hosseinmardi, N. & Javan, M. Prepubertal castration causes the age-dependent changes in hippocampal long-term potentiation. *Synapse* **67**, 235–244 (2013).
63. Chattarji, S., Tomar, A., Suvrathan, A., Ghosh, S. & Rahman, M. M. Neighborhood matters: divergent patterns of stress-induced plasticity across the brain. *Nat. Neurosci.* **18**, 1364–1375 (2015).
64. Panja, D. & Bramham, C. R. BDNF mechanisms in late LTP formation: A synthesis and breakdown. *Neuropharmacology* **76**, 664–676 (2014).
65. Greenberg, G. D. *et al.* Sex differences in stress-induced social withdrawal: role of brain derived neurotrophic factor in the bed nucleus of the stria terminalis. *Front Behav Neurosci* **7**, 223 (2014).
66. Williams, A. V. *et al.* Social approach and social vigilance are differentially regulated by oxytocin receptors in the nucleus accumbens. *Neuropsychopharmacology* 1–8 (2020) doi:10.1038/s41386-020-0657-4.
67. Drickamer, L. C. Seasonal variation in litter size, bodyweight and sexual maturation in juvenile female house mice (Mus musculus). *Lab. Anim.* **11**, 159–162 (1977).
68. Monteiro, L. S. & Falconer, D. S. Compensatory growth and sexual maturity in mice. *Anim. Sci.* **8**, 179–192 (1966).
69. Gubernick, D. J. Reproduction in the California Mouse, Peromyscus californicus. *J. Mammal.* **69**, 857–860 (1988).
70. Sachs, B. D. & Meisel, R. L. Pubertal development of penile reflexes and copulation in male rats. *Psychoneuroendocrinology* **4**, 287–296 (1979).