Sexual differentiation of neural mechanisms of stress sensitivity during puberty

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Abstract

Anxiety disorders are more common in women than men, and this difference arises during puberty. Increased secretion of gonadal hormones during puberty influences brain structure and function, but the extent to which hormones modulate anxiety-related brain circuits is unclear. The slow developing California mouse (*Peromyscus californicus*) is an ideal species for studying the effects of hormones on brain function during adolescence. In adults social defeat stress reduces social approach and increases vigilance in females but not males. Here we show this sex difference is absent in juvenile mice, and that prepubertal castration sensitizes adult males to social defeat. Since adult castration has no effect on stress sensitivity, our data show that gonadal hormones act during puberty to program behavioral responses to stress later in life. In adults, calcium imaging in the medioventral bed nucleus of the stria terminalis shows that threatening social contexts increase calcium transients. Furthermore, prepubertal castration generalizes these responses to less threatening social contexts. Prepubertal treatment with the non-aromatizable androgen dihydrotestosterone acts in males and females to reduce sensitivity to social defeat in adults. Together, these data indicate activation of androgen receptors during puberty are critical for programing behavioral responses to stress in adulthood, highlighting a possible mechanism contributing to sex differences in anxiety.

Significance Statement

Puberty is a key period when sex differences in anxiety emerges. Gonadal hormone release increases during this time but it is largely unknown how they impact brain circuits and behavior. We show that androgens play a key role in programming behavioral responses to social defeat stress. The bed nucleus of the stria terminalis responds to social threats and these responses are more generalized in males without gonadal hormone exposure during puberty. Our findings highlight the importance of pubertal androgens in determining adult behavioral responses to social stress.

Introduction

Adolescence is an important developmental period of cortical and sub-cortical re-organization (1–3) and a period when risk for anxiety disorders increases in girls (4–7). This increased risk is sustained into adulthood (8–13). The increased release of gonadal hormones may modulate these changes (14), as these hormones have important effects on brain structure (15–18) and function (19) by coordinating gene expression (20). Although human imaging studies demonstrate that neural circuits associated with anxiety are altered during pubertal development (21,22), little is known of how gonadal hormones alter stress-sensitivity of these circuits. This is important because stress exposure is an important risk factor for mental illness, and there is strong evidence in preclinical models that androgens can modulate behaviors related to anxiety or depression (23).

Social stressors exert powerful and sex-specific effects on anxiety-related behaviors in rodents (24,25). Female but not male adolescent rats exposed to a combination of social defeat and restraint stress showed reduced sucrose preference and increased passive coping in a forced swim test as adults (26). Adolescent social instability stress, in which rats are repeatedly housed with different cagemates, has stronger effects on anxiety-related behaviors in adult females than males (27,28). In adolescent mice, social isolation enhanced habit-related behaviors to a greater extent in females than males (29). A major outstanding question is whether pubertal hormones drive sex-specific behavioral and neurobiological responses to stress.

An important challenge for studying adolescent development in rodents is the relatively short temporal window. The most common domesticated mouse (30,31) and rat (32) lines are weaned at three weeks of age and are mature by five weeks of age. Here we introduce the California mouse (*Peromyscus californicus*) as a slow developing species that is ideal for studying adolescent development. The California mouse is an important model species for studying sex-specific effects of social stressors on anxiety-related behaviors (33). In adults social defeat increases the responsiveness of the bed nucleus of the stria terminalis (BNST) in adult females (34,35), which drives social avoidance and vigilance in novel social contexts (36–38). In adults these sex differences are independent of gonadal hormones (39,40). Here we use

a combination of pubertal hormone manipulations, calcium imaging, and immunohistochemistry to demonstrate that androgens act during puberty to reduce behavioral responses to social defeat stress. These identify a neuroendocrine mechanism acting during puberty that directs developmental programing of stress-sensitive neural circuits.

Results

Late Onset of Pubertal Development in California mice. Complementary developmental measures suggest a slower developmental trajectory in California mice (Fig. 1A) compared to domesticated mice and rats. In males, testosterone levels increased with age (Fig. 1B, $F_{5,28}$=6.51, p<0.01), but were not significantly elevated until post-natal day 90 (PN90, p<0.001, Cohen's d=1.9). In females, progesterone levels increased with age (Fig. 1B, $F_{5.26}$=2.56, p=0.05) but were not significantly elevated until PN70 (p=0.02, d=1.7). Other developmental measures such as gonadal weight, external genitalia, body weight, and pelage molt followed a similar trajectory (Fig. S1). Together, these data indicate that California mice have an extended period of development, ideal for studying adolescence.

Juvenile Male and Female California Mice Reduce Social and Approach and Increase Vigilance after Defeat. Juvenile mice were exposed to social defeat stress or control conditions after weaning and tested in a social interaction test as PN50 adolescents (Fig. 1C). Social defeat reduced social approach (Fig. 1D, $F_{1,27}$=47.09, p<0.001) in both males (p<0.001, Cohen's d=3.0) and females (p<0.001, d=2.4). Defeated also increased social vigilance (Fig. 1E, Kruskal-Wallis=16.33, p<0.001) in both males (p=0.03, d=1.6) and females (p=0.02, d=1.8). These results contrast with observed sex-differences in adults, which show that social defeat decreases social approach (35,39–41) and increases vigilance (42) in females but not males. During the acclimation phase females spent more time investigating an empty cage than males (Fig. 1F, $F_{1,27}$=6.64, p=0.016) with no effects of stress. There were no differences in locomotor behavior in the open field phase (Fig. 1G), time spent in the center (Fig. S2A) or vigilance behavior during the acclimation phase (Fig S2B). Together with previous work, these results suggest that

89 developmental changes during puberty contribute to sex differences in stress responses in
90 adults.
91
92 **Gonadal Hormones Reduce Susceptibility to Defeat in Adult Males.** To assess the impact of
93 gonadal hormones during puberty, males were assigned to prepubertal castration, sham
94 surgery, or no surgery (Fig. 2A). Gonadectomy affected social approach only in males that were
95 exposed to social defeat as adults (Fig. 2B, trt*stress $F_{2,41}$=3.32, $p<0.05$). Castrated males
96 exposed to defeat had reduced social approach versus controls ($p<0.0001$, d=2.6) whereas
97 there was no effect of defeat in sham or no surgery males. Similarly, social defeat increased
98 vigilance in castrated (Fig. 2C, Mann-Whitney U=23.5, $p<0.01$, d=1.6) but not sham or no
99 surgery mice. There were no differences in behavior during the acclimation (Fig. 2D, S3A) or
100 open field phases (Fig. 2E, S3B). The results contrast sharply with previous results that showed
101 no effect of adult castration or ovariectomy on stress-induced social avoidance in California
102 mice (40), and point to gonadal hormones acting during adolescence as a key mechanism of
103 sexual differentiation. These data suggest that pubertal hormones may play a protective role in
104 organizing the brain to diminish the effects of social defeat on social approach and vigilance.
105 The BNST plays a key role in modulating stress-induced social avoidance and vigilance in males
106 and females (35,38,42). Since prepubertal castration did not affect behavior in unstressed mice,
107 we used fiber photometry to assess effects of castration on BNST neural activity in males
108 exposed to defeat.
109
110 **Calcium imaging of the BNST activity in castrated and intact males.** The BNST modulates social
111 approach and avoidance (34,42), so we performed calcium imaging of GCaMP6f in
112 medioventral BNST to determine how prepubertal castration impacted neural responses in
113 novel social contexts (Fig. 3A, 3B). Previous immediate early gene analyses show that social
114 stress increases activity within the BNST (43,44), but does not have the temporal resolution to
115 determine whether increased activity occurs during brief episodes of social approach or more
116 extended periods of avoidance. Our results indicated that GCaMP signals coincided with social
117 contact with target mice rather than avoidance, regardless of gonadal status. Using DeepLabCut

118 (Fig. 3C, 3D) we determined orientation (45) and distance between focal mice and non-
119 aggressive or aggressive target mice. Increased ΔF/F in BNST was observed when focal mice
120 were within one body length (8 cm) of target mice and within the central (Fig. 3D, 0°≥40°, β= -
121 0.438, z= -9.783, p>0.001) or peripheral visual field (40°≥100°, β= -0.486, z= -10.356, p>0.001),
122 but not if the focal mouse was facing away from target mice (100°≥180°). We then examined
123 ΔF/F in specific behavioral contexts.
124 Intriguingly, the most robust changes in ΔF/F occurred after close contact with an aggressive
125 target mouse. After engaging in nose-to-nose sniffing, both castrated and sham males showed a
126 delayed increase in BNST ΔF/F (Fig. 3E, β=10.22, z=2.05, p=0.04). In contrast, when engaging in
127 nose-to-nose sniffing with a non-aggressive target mouse, castrated males showed a larger
128 increase in BNST ΔF/F than sham males (Fig. 3E, β=13.2, z=2.23, p=0.03). These results suggest
129 that BNST ΔF/F is enhanced by social threats and that this response is more generalized in
130 prepubertally castrated males. We also examined BNST following bouts of freezing, which was
131 robustly induced by aggressive targets (Fig. S4) while non-aggressive targets induced more
132 freezing in castrated males than shams (t_{11}=2.43, p=0.03). There were no acute or delayed
133 changes in ΔF/F following bouts of freezing (Fig. 3F). We next examined bouts of anogenital
134 sniffing, which were less frequent and limited to non-aggressive target mice (Fig. S4). Similar to
135 nose-to-nose sniffing castrated mice showed a larger delayed increase in ΔF/F in BNST than
136 shams (Fig. 3G, β=6.12, z=2.06, p=0.04. Finally, when castrated males were attacked by
137 aggressive target mice, there were acute (Fig. 3H, β=5.71, z=2.56, p=0.01) and delayed (β=5.71,
138 z=2.67, p=0.008) increases in ΔF/F. Together these data suggest that neuronal activity in BNST
139 increases after potentially-threatening social situations, and is generalized to less threatening
140 contexts after prepubertal castration. To assess the extent to which prepubertal castration
141 impacted neuronal activity in other circuits modulating social approach, we used c-fos
142 immunohistochemistry.
143
144 **Gonadal Hormones Reduce Neural Activity in PVN Oxytocin Neurons**: Social defeat stress has
145 been demonstrated to elevated the activity of oxytocin releasing neurons in PVN (34), a
146 response that has been associated with heightened activation of stress-reactive neurons

147 expressing oxytocin receptors in the anteromedial BNST (42). We used oxytocin/c-fos
148 immunohistochemistry (Fig. 4A,B) to examine anterior and posterior PVN, which differ in
149 connectivity (46) and stress sensitivity (34). In anterior PVN, prepubertal castration increased
150 oxytocin/c-fos colocalizations when compared to sham (Fig. 4C', Mann-Whitney U=38, p=0.01)
151 in mice that had undergone social defeat. Oxytocin/c-fos colocalizations were negatively
152 correlated with social approach (Fig. 4C", Spearman ρ=-0.55, p<0.01) and positively correlated
153 with social vigilance (Fig. S5A, ρ=0.59, p<0.01). In the posterior PVN castration increased
154 oxytocin/c-fos colocalizations regardless of stress status (Fig. 4D', Mann-Whitney U=113.5,
155 p=0.04) and oxytocin/c-fos colocalizations was negatively correlated with social approach (Fig.
156 4D", ρ=-0.41, p=0.04) but not vigilance (Fig. S5B). We also examined the effects of prepubertal
157 castration on c-fos in the anteromedial BNST, where oxytocin induces social avoidance and
158 vigilance (38). Prepubertal castration increased the number of c-fos positive neurons in
159 BNSTam (Fig. 4E', S5D, $F_{1,21}$=4.51, p=0.046), regardless of stress status. The number of c-fos cells
160 was negatively correlated with social approach (Fig. 4E", ρ=-0.46, p=0.02) but not vigilance (Fig.
161 S5C).
162 We also tested whether defeat induced social avoidance and vigilance in adolescent mice
163 was dependent on oxytocin receptors (Fig. 4F) as in adults. In both males and females,
164 treatment with 5 mg/kg i.p. of the oxytocin receptor antagonist L-368,899 30 min before testing
165 increased social approach (Fig. 4G, main effect of dose $F_{2,42}$=8.42, p<0.001) and decreased
166 vigilance (Fig. 4H, Kruskal-Wallis=15.36, p<0.009). There were no differences in behavior during
167 the acclimation (Fig. S5E,S5F) or open field (Fig. S5G, S5H) phases. Together these results
168 suggest that stress-induced social avoidance and vigilance in both male and female juvenile
169 mice is dependent on oxytocin receptor activation, as seen in adult females. Additionally, the
170 results indicate that male pubertal hormones program these circuits to be less active in
171 adulthood.
172
173 **Androgens Reverse the Effects of Prepubertal Castration on Behavior**.
174 To test the impact of androgen replacement at puberty, juvenile males were prepubertally
175 castrated and randomly assigned to receive a silastic implant containing testosterone, the non-

176 aromatizable androgen dihydrotestosterone (DHT), or sealant only (Fig 5A). These implants
177 produce plasma hormone levels within physiological range of adult male California mice (47). At
178 PN90, mice were exposed to defeat stress and then tested in a social interaction test. Results
179 demonstrated that hormone replacement altered social approach (Fig. 5B, $F_{2,22}$=6.2, p<0.01)
180 and vigilance (Fig. 5C, Kruskal-Wallis=13.13, p<0.001). For social approach both testosterone
181 (p=0.002, d=1.7) and DHT (p=0.002, d=1.0) treatment yielded significantly higher levels of social
182 approach compared to males treated with empty implants. Similarly for vigilance both
183 testosterone (p<0.001, d=3.1) and DHT (P=0.049, d=1.1) treated males had lower vigilance than
184 males treated with empty implants. There was a nonsignificant trend for testosterone treated
185 males to have lower vigilance than DHT treated males (p=0.078, d=1.0). During the acclimation
186 phase T and DHT treatment increased approach to an empty cage (Fig. S6A, $F_{2,22}$=5.02, p<0.02)
187 and there were no differences in behavior during the open field phase (Fig. S6B, S6C). We also
188 tested whether DHT treatment at puberty could impact female behavior (Fig. 5D). In social
189 interaction tests performed before stress exposure, there were no differences in social
190 approach (Fig. 5E), social vigilance (Fig. 5F), acclimation (Fig. S6D) or open field behavior (Fig.
191 S6E,F). Importantly, after social defeat, hormone treatment altered social approach (Fig. 5E,
192 $F_{2,23}$=3.76, p= 0.04) with DHT increasing social approach versus empty implants (p=0.02, d=0.1).
193 Effects on vigilance were weaker (Kruskal-Wallis=5.04, p=0.08), with a post-hoc test indicating
194 lower levels of vigilance in DHT treated mice versus empty implant (Mann-Whitney=11,
195 p=0.028, d=1.2). After stress exposure DHT treatment increased approach to an empty cage
196 (Fig. S6D, $F_{2,23}$=5.32, p= 0.01) while there were no differences in the open field phase (Fig. S6E,
197 S6F)
198
199 **Discussion**
200
201 Sex differences in stress-sensitivity emerge at puberty in both humans (48,49) and animals (26–
202 28), coinciding with an increased incidence of anxiety in women (4,6,7,12). However, little is
203 known about the underlying mechanisms. Using the California mouse, we found stress-induced
204 social avoidance and vigilance occurs in both sexes before puberty. Prepubertal castration

205 enhanced behavioral responses to stress in adult males. Gonadal hormones do not affect social
206 avoidance in adults (40,50), suggesting that puberty is a critical period for sexual differentiation.
207 Dihydrotestosterone replacement during puberty in castrated males and intact females
208 reduced stress-induced social avoidance in adulthood, highlighting an essential role for
209 androgen receptors. Using real-time calcium imaging we found that neurons in ventral BNST are
210 highly responsive to threatening social contexts, and that prepubertal castration generalized
211 these responses to non-aggressive mice. Our findings indicate that androgens play an
212 organizational role during puberty, which attenuates behavioral and neural responses to social
213 stress in adulthood.
214
215 **BNST neurons respond to social contact**. Immediate early gene analyses in rodents (34,51–54)
216 and neuroimaging studies in primates (55) and humans (56) show that stressful social contexts
217 increase neural activity within the BNST. However, these analyses lack the temporal resolution
218 to associate changes in neural activity with specific behaviors. Our calcium imaging data show
219 that ventral BNST calcium transients occurred after close contact with target mice. Specifically,
220 increased activity was observed following active engagement with aggressive target mice, such
221 as nose-to-nose sniffing or aggression. Intriguingly no increases occurred during freezing, a
222 behavior that does not involve active social engagement. While it has been hypothesized that
223 the BNST encodes more distant threats (57), our results are more consistent with recent
224 hypotheses that the BNST is highly responsive to proximate threats (58). The strength of
225 calcium transients in the presence of non-aggressive target mice was dependent on exposure to
226 pubertal hormones.
227 Pre-pubertally castrated males exhibited more freezing and heightened BNST calcium
228 transients in the presence of non-aggressive target mice. These results are consistent with our
229 findings showing that pre-pubertal castration leads to increased avoidance and vigilance
230 towards unfamiliar target mice, similar to stressed adult females (40,59). These findings suggest
231 that the absence of male gonadal hormones during puberty may alter valence assessment of
232 novel social contexts (60,61), potentially leading to an over-generalization of threat, which is a
233 key characteristic of anxiety disorders (62). Our results align with imaging studies in nonhuman

234 primates (63) and humans (64) reporting stronger BNST responses in individuals with elevated
235 anxiety-related behaviors. Intriguingly, androgens can reduce neuronal excitability in both adult
236 (65) and adolescent rodents (66–68). These findings are in line with our observations that
237 prepubertal castration led to increased c-fos/oxytocin colocalizations in the PVN and c-fos
238 expression in anteromedial BNST. Taken together, our results suggest that androgens may play
239 a role during puberty to reduce the excitability of circuits that are affected by social defeat.
240 The question remains as to which BNST cell types respond to social threats. This is further
241 complicated by the fact that the BNST exhibits heterogeneity in neural responses, even within
242 genetically defined cell populations (69–71). This variation could be explained by projection-
243 specific populations within a cell-type (72). This is supported by optogenetic studies showing
244 the projection-specific effects of BNST neurons on behavior (73–76). Future research
245 considering genetic and projection-specific populations during neuronal recording of activity in
246 different social contexts should be highly informative.
247
248 **Activation of androgen receptors during puberty blunts stress induced social avoidance**. In
249 adult hamsters, dominant males have more androgen receptor expression in the medial
250 amygdala (MEA) than subordinates and pharmacological inhibition of AR in the MEA of
251 dominant males increases sensitivity to social stress (77). Similarly testosterone can exert acute
252 anxiolytic effects in a variety of behavioral assays (78–80). Interestingly, in adult California mice,
253 androgens did not appear to affect social approach regardless of stress exposure (40). Here we
254 show that androgens act during puberty to influence how social behavior is impacted by stress,
255 suggesting that even in species where androgens do not have an overt role in regulating stress-
256 sensitivity in adults, androgens can program behavioral responses during adolescence. This may
257 be particularly relevant for humans, where the relationship between gonadal hormones and
258 stress responses in adults can be inconsistent (81–83). Although adolescent DHT and
259 testosterone treatment had similar effects on social approach in stressed males, the effect of
260 DHT on vigilance was weaker. Similar effects were observed in females. These results suggest
261 that estrogen receptors may play a complementary role in pubertal organization of stress-
262 induced behavior. Estrogens can enhance anxiety-related behaviors by activating *Esr1* or exert

263 anxiolytic effects via *Esr2* (84). Both receptors are present in the BNST, and future studies
264 should consider the possible developmental effects of these receptors on stress-induced social
265 vigilance.
266
267 **Functional implications**
268 Steroid hormones alter brain development during puberty (15,17,19), yet how these changes
269 affect anxiety-related behavior has been unclear. We showed that sex differences in social
270 stress effects on behavior are driven, in part, by pubertal androgen exposure in males. A lack of
271 pubertal hormone exposure increased reactivity of BNST neurons during social engagement.
272 Interestingly, the effects pubertal androgens on social behavior were only apparent following
273 exposure to social defeat. This demonstrates that organizational effects of pubertal hormones
274 can cause latent vulnerabilities that are only revealed after stressful social experiences. Our
275 results suggest that it will be worthwhile to consider whether testosterone levels during
276 adolescence predict behavioral or neural responses to stressors in adulthood. Overall, our
277 research sheds light on how androgens shape the complex interplay between brain circuits and
278 behavioral sensitivity to stress.
279
280 **Methods**
281
282 <u>Animals</u>
283
284 All studies were conducted with California mice (*Peromyscus californicus*) raised in a colony at
285 UC Davis. Mice were housed in same sex groups (2-4) in clear polypropylene cages with Sani-
286 Chip bedding (Harlan Laboratories, Indianapolis, IN, USA), Nestlets (Ancare, Bellmore, NY, USA)
287 and Enviro-Dri (Eco-bedding, Fibercore, Cleveland, OH, USA). Mice were kept on a 16L:8D light
288 cycle and had *ad libitum* access to food and water. All procedures were approved by the
289 Institutional Animal Care and Use Committee at the University of California, Davis and in
290 accordance with National Institutes of Health (NIH) guidelines.
291

292 Puberty Quantification

293 For measures of first vaginal opening, preputial separation, weight, and coat color mice were
294 briefly anesthetized (>1 minute isoflurane) before being weighed, photographed, and assessed
295 for vaginal opening/preputial separation. A separate set of mice were euthanized to determine
296 uterine or testes size. Trunk blood was collected and plasma obtained and frozen for hormone
297 analyses. Plasma was extracted and hormones quantified on a QTRAP 550 quadruple linear ion
298 trap mass spectrometer equipped with an atmospheric pressure chemical ionization source (LC-
299 MS/MS) as previously described (85). Quantitative results were recorded as multiple reaction
300 monitoring (MRM) area counts after determination for the response factor for each hormone
301 and internal standard. We also quantified the transition from the juvenile pelage (dark gray) to
302 adult (brown) adult pelage (86) by quantifying digital images in ImageJ as previously described
303 (87).

304

305 Juvenile Social Defeat

306 Male and female juveniles (PN34-36) underwent 3 consecutive days of social defeat stress
307 (or control handling) as described previously (50). During each episode of defeat the focal
308 mouse was placed in the home cage of a novel male-female adult resident pair. The opposite
309 sex resident mouse was removed from the cage prior to the start of the defeat session. Each
310 session of defeat lasted for 7 minutes, or until the test mouse received 7 bites. Control mice
311 were placed into a clean, empty cage for 7 minutes across 3 consecutive days.

312 On PN50 mice underwent a social interaction test. The social interaction test had 3 phases:
313 open field, acclimation, and interaction. In the open field phase, the mouse was placed into an
314 open area and allowed to explore it for 3 minutes. In the acclimation phase, an empty cage was
315 placed at one end of the arena and the mouse was allowed to explore for 3 minutes. In the
316 interaction phase, a caged, unknown adult conspecific replaced the empty cage at one end of
317 the arena, and the mouse was allowed to explore for 3 minutes. Time in the interaction zone (8
318 cm from the cage) is defined as “social approach” was scored with AnyMaze software (40).
319 Duration of vigilance behavior was hand scored from a video recording. Vigilance was defined

320 as any time the test mouse was sitting still, head oriented toward the target mouse, while
321 outside of the interaction zone (37).
322
323 Prepubertal Castration
324 Male juveniles were randomly assigned to be castrated, sham surgery, or no surgery control
325 between PN35 and PN40 (47). During castration mice were anesthetized with isoflurane and
326 treated with 0.1mg/kg buprenorphine and 5mg/kg of carprofen. Non-surgery controls were
327 included to control for possible effects of early life exposure to the anesthesia isoflurane (88).
328 These mice received no manipulations until the start of social defeat stress/control handling. At
329 PN90-92 all mice underwent 3 days of either social defeat or control handling. At PN106 mice
330 were tested in a social interaction test.
331
332 Calcium imaging of the BNST activity in castrated and intact males
333
334 Male juveniles were randomly assigned to castration or sham surgery after weaning. As
335 adults, all mice received an injection of AAV9.Syn.GCaMP6s at a rate of 100nL/min for a total
336 volume of 500nL in the ventral BNST (AP +0.45mm, ML +/-1.0mm, DV -5.6mm). The virus was
337 allowed to diffuse for 10 minutes before the needle was withdrawn. An optical fiber (Doric)
338 with 2.5 mm core and 0.66 NA threaded through a ceramic ferrule was implanted at the
339 injection site and the ferrule was secured to the skull with a layer of C&B Metabond (Parkell),
340 followed by a layer of dental cement to form a thick headcap. Mice were housed two per cage
341 with a clear, perforated acrylic divider that allowed auditory, tactile and olfactory contact.
342 Mice recovered for 1 week before undergoing 3 consecutive days of social defeat stress. Ten
343 days later mice began 3 consecutive days of patch cord habituation in which the patch cord was
344 gently coupled to their optical fiber implant. The mouse then explored a novel cage for 10 min.
345 The next day mice were tested in a social interaction test with freely moving target mice. The
346 focal mouse was placed into an empty area (51x25.4x76 cm) attached to a small box (13x10x18
347 cm) with a sliding door for 6 min. Photometry recording was performed with an isosbestic
348 channel of 405nm and an excitatory channel of 470nm, both set to 50µW output. Photometry

349 data from the acclimation period were excluded from analysis due to initial bleaching. Next, an
350 unfamiliar, nonaggressive adult male target mouse was introduced into the arena through the
351 sliding door. Mice were allowed to freely interact for 3 min. The target mouse was removed
352 and then a sexually experienced, aggressive male (from a previous social defeat episode) was
353 introduced into the arena for 3 min. After testing brains were collected to confirm viral
354 expression and placement of the fiber.

355 We used Deeplabcut to quantify distance and orientation of the focal mouse in relation to
356 target mice with precision that matched the high temporal resolution of photometry data (89–
357 91). Video was taken from the side view at a rate of 30fps. And training was performed to track
358 the nose, right ear, left ear, body midpoint, front leg, back leg, tail base, and tail tip for both the
359 test mouse and stimulus mouse. The tracking for the nose and body midpoint were used in
360 order to determine proximity, defined as times when the body midpoint of the two mice were
361 within 8 cm (the approximate length of a California mouse) of each other. We determined the
362 direction the test mouse was facing in relation to the stimulus mouse using their coordinate
363 points obtained from the deeplabcut tracking (test mouse nose, body midpoint, and target
364 mouse nose). We binned angles as within the test mouse's central vision (0°≥40°), peripheral
365 vision (40°≥100°), or facing away from the stimulus mouse (100°≥180°) (45). All scripts are
366 deposited at https://github.com/bctrainorlab/behavioral_quantification. Nose to nose sniffing,
367 anogenital sniffing, freezing and aggressive behavior directed towards focal mice were scored
368 by an observer without knowledge of the treatment group using BORIS.

369

370 Pubertal hormone manipulation studies

371 Male juveniles were castrated and received a subcutaneous silastic implant (i.d. 0.04 in, o.d.
372 0.085 in) containing 1mm crystalline testosterone, dihydrotestosterone, or Dowsil Sealant
373 (3145 RTV MIL-A-46146). These implants yield hormone levels that are within the physiological
374 range of intact male California mice (92). At adulthood mice underwent 3 days social defeat and
375 were tested in a social interaction test two weeks later.

376 Intact females were randomly assigned to receive a DHT or empty implant at PN35-PN40 as
377 described for males. At PN90 all mice were tested in a social interaction test. Three days later

378 females were exposed to 3 days of social defeat on consecutive days. One week later females
379 were tested again in a social interaction test. This within-subjects design produces behavioral
380 effects similar to the between-subjects design used for males (93).
381
382 Immunohistochemistry
383
384 Sections from castrated or intact males were stained for either oxytocin/c-fos (PVN) or c-fos
385 (anteromedial BNST) as previously described(34). Sections were blocked in 10% normal goat
386 serum for 20 minutes and then incubated in rabbit anti-cfos (Synaptic Systems, 1:K) in PBS 0.5%
387 TritonX overnight at room temperature. For oxytocin double staining, sections were washed in
388 PBS and then incubated in goat anti-rabbit AlexaFluor 555 (Molecular Probes, 1:500 in PBX-TX)
389 for 2 hrs. Sections were then incubated in mouse anti-oxytocin (Sigma-Aldrich MAB5296 1:2000
390 in PBS-TX) overnight at 4 C. Sections were then washed in PBS and incubated goat anti-mouse
391 AlexaFluor 488 (Molecular Probes, 1:500 in PBS-TX) for 2 hrs. For BNSTam sections, sections
392 were washed in PBS and incubated in biotinylated goat anti-rabbit (Vector labs, 1:500 in PBS-TX
393 for 2 hrs). Sections were washed in PBS and then incubated in avidin-conjugated peroxidase
394 (Vector labs) for 30 min. After washing in PBS sections were developed in nickel enhanced
395 diaminobenzidine (Vector labs) for 2 min.
396
397 Juvenile Social Defeat + OTA Injections
398
399 Male and female juveniles (PN34-36) underwent 3 consecutive days of social defeat stress.
400 At P50 mice were tested in a social interaction test. 30 minutes before the start of the social
401 interaction test, each mouse received an intraperitoneal (i.p.) injection of 5mg/kg or 10mg/kg
402 of the oxytocin antagonist (OTA) L-368,899 (37), or an injection of sterile phosphate buffered
403 saline (PBS).
404
405 Statistics
406

Statistical analyses for experiments (excluding fiber photometry experiments) were performed using R statistical software. Normality was assessed via QQPlot. A Flinger-Killen test was used to assess homogeneity of variance. Steroid hormone data were log transformed for analysis. Hormone and most behavioral and cell count data were analyzed using ANOVA with planned comparisons for comparing treatment groups with controls. Social vigilance and oxytocin/c-fos colocalization data had heterogeneous variability, so non-parametric analyses were used.

Photometry data was analyzed using a custom Python script, down sampled to 30 samples per second to match the 30fps video framerate. 405nm was used as the isosbestic wavelength and 470nm as the excitatory wavelength. To correct for motion artifact and bleaching of the fluorophore, the 405nm signal was fit to a biexponential model and subtracted from the excitatory output. Such that ΔF/F = [100*((470nm signal – fitted signal)/fitted signal)]. Z-scores were calculated using the formula: z-score = ((ΔF/F - mean ΔF/F of baseline period)/standard deviation of ΔF/F of baseline period). For behavior non-specific analysis, the baseline period was 5 to 25 seconds after recording started. For behavior-specific analysis, the baseline period was -10 to -5 seconds before the behavior onset. Area under the curve (AUC) was calculated using the trapezoidal method for specified time points across behaviors. Linear mixed effects models were used to determine the effects of surgery, social condition, and time point using the statsmodels package (94) in Python using one of the following formulas:

- Z-Score=surgery condition + social condition + distance + surgery condition*social condition + surgery condition*distance + social condition*distance + surgery condition*social condition*distance + (1|mouse)
- AUC=surgery condition + time point + surgery condition*time point + (1|mouse)
- AUC=social condition + time point + social condition*time point + (1|mouse)
- AUC=time point + (1|mouse)

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Figures and Legends

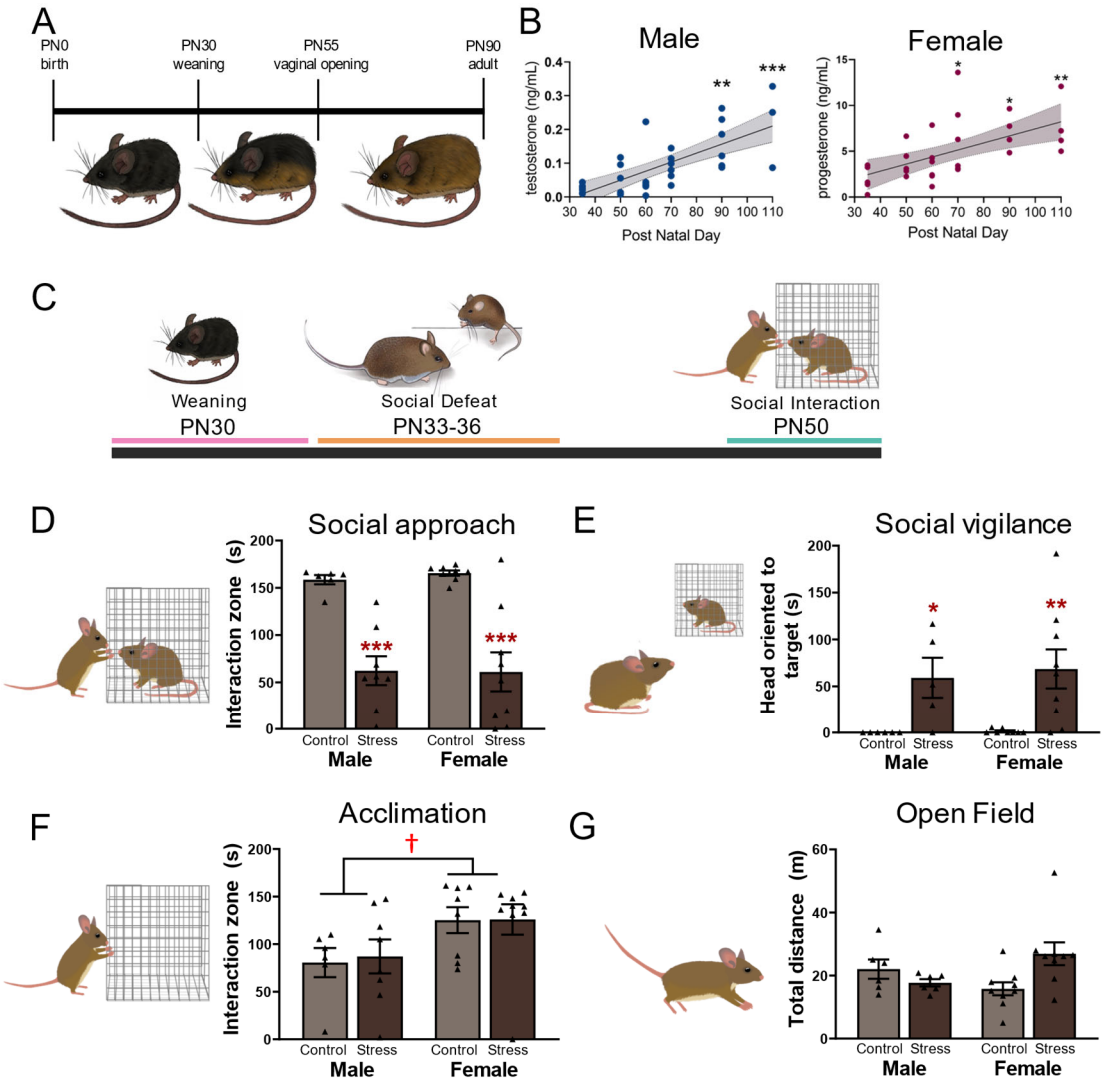

Figure 1: Delayed puberty in California mice is a period of sexual differentiation of behavioral stress responses. A. Summary of pubertal development in California mice **B.** In males testosterone levels are not increased until postnatal day (PN) 90 while in females progesterone levels increase at PN70 (n=4-5 per time point). **C.** Prepubertal California mice were exposed to social defeat or control conditions after weaning and tested as juveniles at PN50. **D,E.** Social defeat reduced social approach and increased social vigilance in both males and females (n=6-8 per group). **F.** Females investigated an empty cage during the acclimation phase more than males. **G.** There were no differences in locomotor behavior in the open field phase. *, **, *** $p<0.05$, $p<0.01$, $p<0.001$ vs. PN 35. *, **, *** $p<0.05$, $p<0.01$, $p<0.001$ vs same sex control. †$p < 0.05$ vs. males.

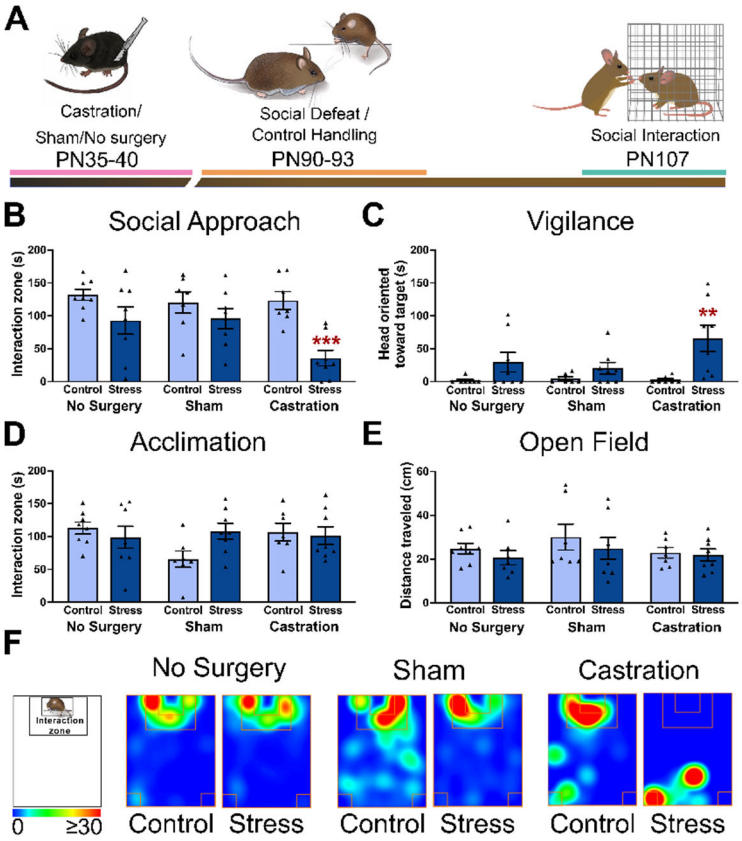

Figure 2: Prepubertal castration increases sensitivity to social defeat in adulthood. A. Timeline of prepubertal castration and behavioral testing in adults. **B,C.** Social defeat decreased social approach and increased social vigilance in castrated males but not sham or no surgery controls (n=7-8 per group). **D,E.** No differences were observed during the acclimation of open field phase. **F.** Representative heatmaps for the interaction phase showing reduced time spent in the interaction zone castrated males exposed to social defeat. **, *** $p<0.01$, $p<0.001$ vs control.

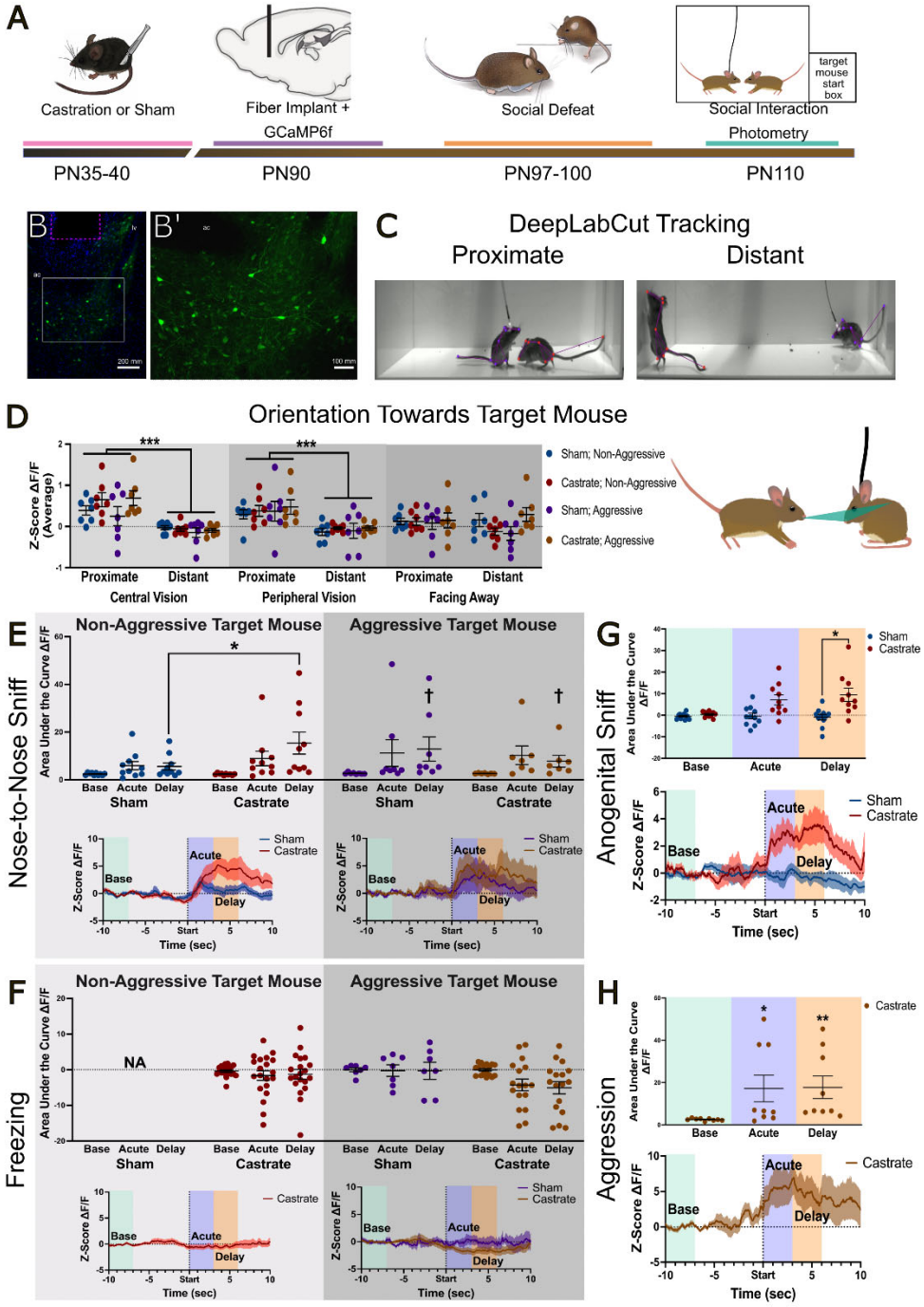

Figure 3: Prepubertal castration increases BNST neural activity in sensitivity to social defeat in adulthood. A. Experimental timeline for fiber photometry observations of GCaMP6f in the ventral BNST of prepubertally castrated or sham surgery California mice. **B.** Photomicrographs of GCaMP in BNST at low (B) and high (B') magnification. The magenta box indicates position of the fiber. **C.** Representative images showing DeepLabCut tracking of mice in proximate and distant conditions. **D.** DeepLabCut tracking of body midpoint and nose of the focal mouse was used to determine the orientation of the focal mouse to the target mouse nose (green triangle).

GCaMP6f signals were significantly stronger when the focal mouse was within 8 cm of the target mouse but only when the target mouse was in central vision (0°≥40°) or peripheral vision (40°≥100°). **E.** Nose-to-nose sniffing with a non-aggressive target mouse induced a delayed increase in ΔF/F in castrated but not sham males. In contract nose-to-nose sniffing with an aggressive target mouse increased ΔF/F in both sham and castrated males. **F.** There were no changes in ΔF/F following bouts of freezing. **G.** Anogenital sniffing of non-aggressive target mice increased ΔF/F in castrated but not sham males. **H.** When castrated males were attacked by aggressive target mice, increased ΔF/F was observed. Sham males were not attacked enough for analysis. * $p< 0.05$, ** $p<0.01$ vs sham. *** $p<0.001$ vs proximate, † $p<0.05$ vs. baseline. ac= anterior commissure.

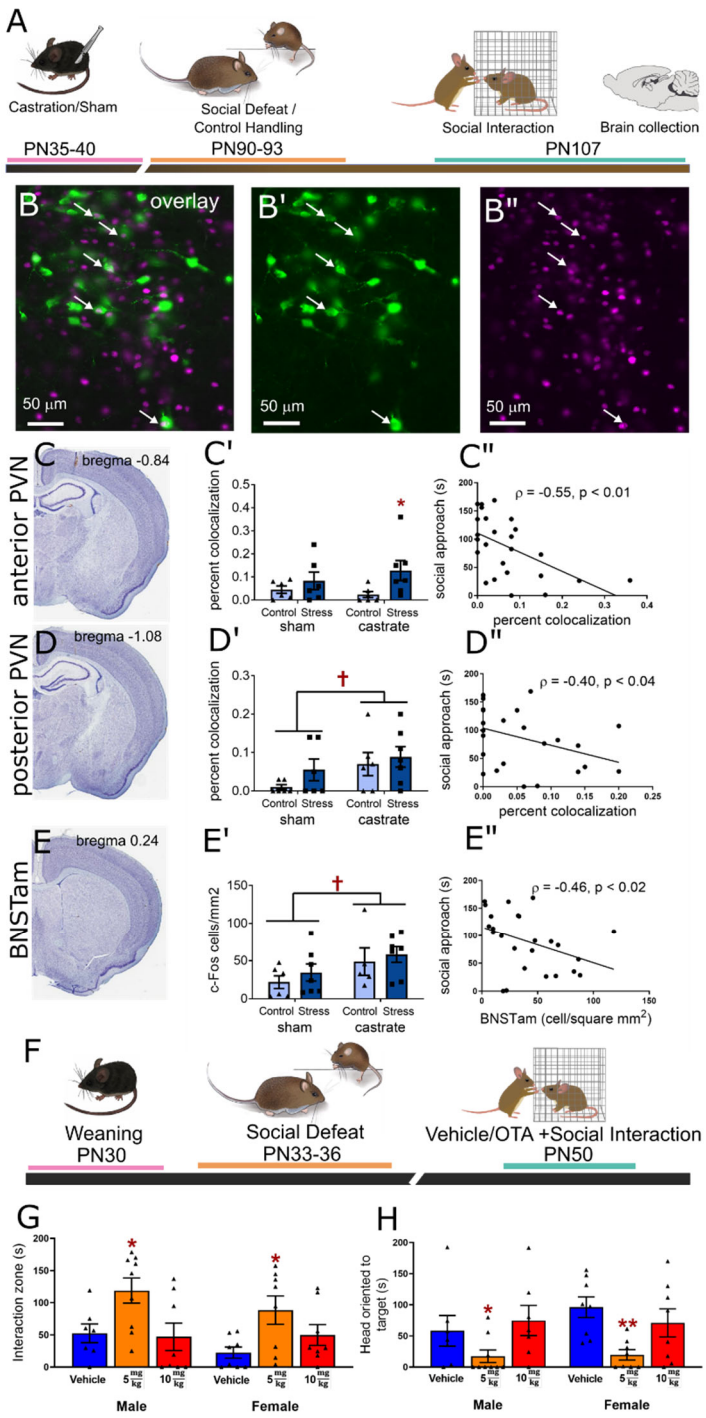

Figure 4: Sexual differentiation of oxytocin-dependent circuits of social avoidance occurs during puberty. A. Experimental timeline for immunohistochemistry analyses in prepubertally castrated or intact California mice. **B.** Overlay of oxytocin (green) and c-fos (magenta) immunostaining in the paraventricular nucleus. Arrows indicate colocalizations in oxytocin (B') and c-fos (B") images. **C.** In the anterior PVN social defeat increased colocalizations (n=6-7 per group) in prepubertally castrated males but not intact males(C') and that social approach was negatively correlated with oxytocin/c-fos colocalizations (C"). **D**. In the posterior PVN castration increased oxytocin/c-fos colocalizations regardless of stress status (D') and social approach was negatively correlated with colocalizations. E. In anteromedial BNST, where oxytocin receptors drive social avoidance, prepubertal castration increased c-fos immunoreactivity regardless of stress status (E') and c-fos positive cells were negatively correlated with social approach (E"). **F**. Experimental timeline for examining effects of oxytocin receptors on social behavior. **G,H**. In both males and females exposed to social defeat an i.p. injection of 5 mg/kg of the oxytocin receptor antagonist L368,899 increased social approach and decreased social vigilance (n=7-9 per group). *, ** $p<0.05$, control/vehicle. †$p < 0.05$ vs. intact.

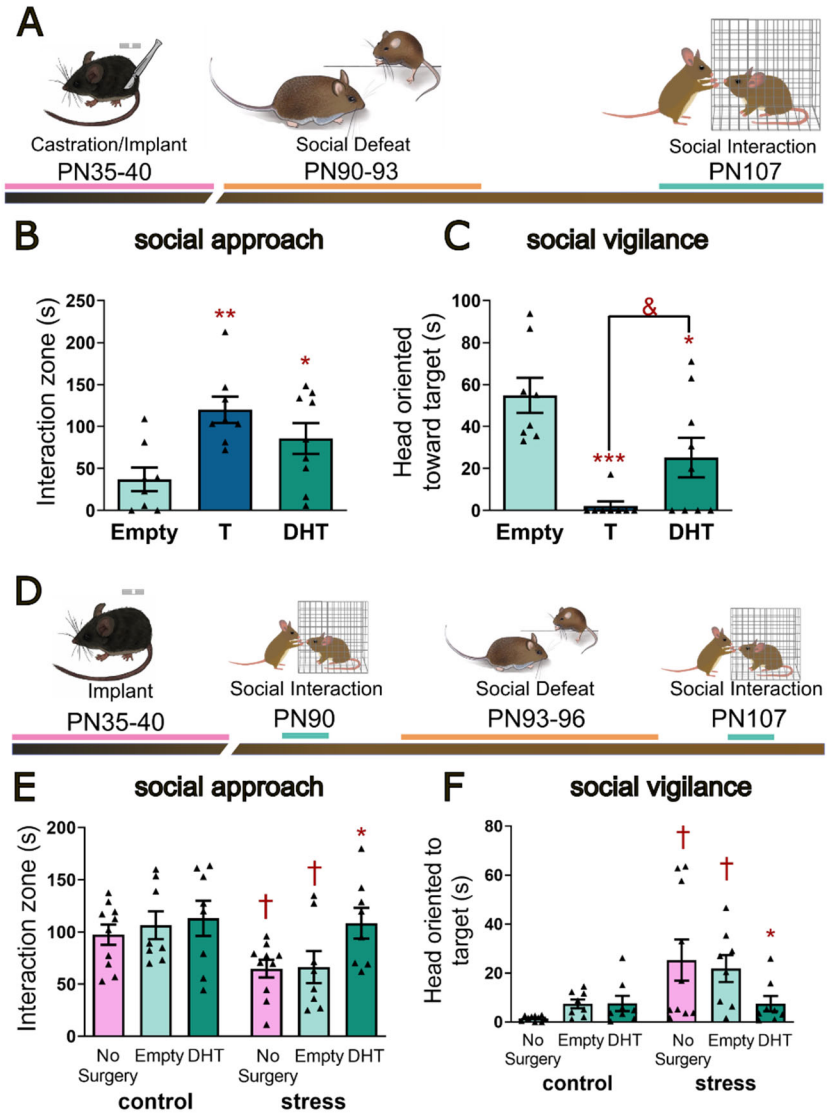

Figure 5: Androgen treatment at puberty reduces effects of social defeat on social approach and vigilance in males and females. A. Experimental timeline for surgery, social defeat, and social interaction testing in males. **B**. Castrated males treated with dihydrotestosterone (DHT) or testosterone (T) implants had higher social approach than males treated with empty implants (n=7-8 per group). **C**. Treatment with DHT or T also lowered social vigilance. **D**. Experimental timeline for surgery, behavior testing, and social defeat in female California mice. **E**. Hormone treatment had no effect on social approach before stress exposure but after stress exposure DHT treated females showed more social approach than females treated with empty implants or no surgery controls (n=8-10 per group). **F**. Similarly there were no differences in social vigilance in before stress but after stress social vigilance increased in no surgery control and empty implant females but not DHT females. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. empty implant. & $p<0.05$ vs T. †$p < 0.05$ vs. control (pre-stress).