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Stress during puberty exerts sex-specific effects on depressive-like behavior and monoamine neurotransmitters in adolescence and adulthood☆

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ABSTRACT

Psychiatric disorders including major depression are twice as prevalent in women compared to men. This sex difference in prevalence only emerges after the onset of puberty, suggesting that puberty may be a sensitive period during which sex-associated vulnerability to stress-related depression might become established. Thus, this study investigated whether stress occurring specifically during the pubertal window of adolescence may be responsible for this sex difference in depression vulnerability. Male and female rats were exposed to a three-day stress protocol during puberty (postnatal days 35–37 in females, 45–47 in males) and underwent behavioral tests in adolescence or adulthood measuring anhedonia, anxiety-like behavior, locomotor activity and antidepressant-like behavior. Brainstem and striatum tissue were collected from a separate cohort of behavioral test-naïve rats in adolescence or adulthood to quantify the effect of pubertal stress on monoamine neurotransmitters. Pubertal stress increased immobility behavior in the forced swim test in both sexes in adolescence and adulthood. In adolescence, pubertal stress altered escape-oriented behaviors in a sex-specific manner: decreasing climbing in males but not females and decreasing swimming in females but not males. Pubertal stress decreased adolescent brainstem noradrenaline specifically in females and had opposing effects in adolescent males and females on brainstem serotonin turnover. Pubertal stress induced anhedonia in the saccharin preference test in adult males but not females, an effect paralleled by a male-specific decrease in striatal dopamine turnover. Pubertal stress did not significantly impact anxiety-like behavior or locomotor activity in any sex at either age. Taken together, these data suggest that although pubertal stress did not preferentially increase female vulnerability to depressive-like behaviors compared to males, stress during puberty exerts sex-specific effects on depressive-like behavior and anhedonia, possibly through discrete neurotransmitter systems.

1. Introduction

Puberty is a period characterized by substantial physical, hormonal, and psychological changes (Spear, 2000). During this critical window of development, the brain is particularly sensitive to stressful events (Romeo et al., 2016). Stress, particularly during early life, is a risk factor for the development of psychiatric disorders (Gershon et al., 2013; Heim et al., 2008; Kessler et al., 1997). Stress-related psychiatric disorders like

Abbreviations: Corticotropin releasing factor, (CRF); 3,4-Dihydroxyphenylacetic acid, (DOPAC); 5-hydroxyindoleacetic acid, (5-HIAA); 5-hydroxytryptamine, (5-HT); Elevated plus maze, (EPM); forced swim test, (FST); female urine sniffing test, (FUST); hypothalamic–pituitary–adrenal, (HPA); high-performance liquid chromatography, (HPLC); novelty-induced hypophagia, (NIH); novelty-suppressed feeding, (NSF); open field test, (OFT); prefrontal cortex, (PFC); postnatal day, (PND); post-traumatic stress disorder, (PTSD); saccharin preference test, (SPT); selective serotonin reuptake inhibitors, (SSRIs).

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depression and post-traumatic stress disorder (PTSD) are twice as prevalent in women compared to men (Kessler et al., 1993; Kuehner, 2017; Seedat et al., 2009; World Health Organization, 2017). Interestingly, this difference in prevalence of depression only emerges after the onset of puberty, suggesting that puberty may be a sensitive period during which sex-associated vulnerability to stress-related disorders might become established (Kessler et al., 1993). However, to date, no preclinical studies have investigated whether stress applied specifically during the short pubertal window of adolescence exerts sex-specific effects on brain and behavior.

Sex differences in depression prevalence emerge during puberty and persist throughout the reproductive years (Angold and Costello, 2006; Gutiérrez-Lobos et al., 2002; Kessler et al., 1993). Despite this sex difference in prevalence of stress-related psychiatric disorders, many preclinical studies of stress in rodents have neglected to study both males and females or do not include sex as a biological variable (Mamlouk et al., 2020; Shansky and Murphy, 2021). Furthermore, no preclinical studies have yet investigated whether stress occurring specifically during the pubertal period of adolescence of each sex might contribute to sex differences in vulnerability to depression or other stress-related psychiatric disorders. As a result, the neurobehavioral ramifications of stress occurring during puberty in both males and females are incompletely understood.

The neuroendocrine changes that characterize puberty signal the beginning of adolescence, the gradual transition from childhood to adulthood (Foilb et al., 2011). During adolescence, substantial remodeling and maturation takes place in brain areas involved in regulating the stress response and emotional processing such as the prefrontal cortex, hippocampus, and amygdala (Spear, 2000). Adolescence is a critical transition in hypothalamic–pituitary–adrenal (HPA) axis development from the lengthy, exaggerated stress response of the pre-pubertal period, to a typical adult stress response (Green and McCormick, 2016; Klein and Romeo, 2013; Romeo, 2018). Rapid structural changes mediated in part by activational effects of gonadal hormones during puberty may contribute to a vulnerability to stressors, particularly as brain areas involved in HPA axis regulation show relatively high levels of corticosteroid receptors during adolescence (Eiland and Romeo, 2013; Pryce, 2008). The transition from adolescence to adulthood is also marked by the maturation of dopamine, noradrenaline, and serotonin systems, monoamine neurotransmitters that are implicated in the regulation of reward, mood, and anxiety (O'Leary et al., 2020; Pitzer, 2019). Disruption of the development of monoamine neurotransmitters systems by stress during puberty could perhaps contribute to behavioral alterations later in adult life (Andersen and Teicher, 2008; McCormick and Green, 2013).

Previous research in rodents has indicated that exposure to stress during the broader period of adolescence has an impact on physiology and behavior into adulthood (Reviewed in Hollis et al., 2013; McCormick et al., 2010). Adolescent stress has been shown to impact the functioning of key brain areas involved in dopaminergic, serotonergic, and noradrenergic circuits (Bingham et al., 2011; Borodovitsyna et al., 2018; Burke et al., 2017; Forster et al., 2018; Novick et al., 2011). For example, adolescent social defeat in male rats (PND 35–40) increased dopamine transporter binding in the mPFC and increased D1 receptor binding in the striatum in adulthood (Novick et al., 2011). However, to the best of our knowledge, no studies have limited the stress exposure specifically to just the pubertal onset and maturation time window which, in both humans and rodents, occurs at an earlier chronological in females than males (Bell, 2018; Lewis et al., 2002). Therefore, the pubertal window in males and females may correspond to subtly different periods of neuronal maturation and development leading to unique vulnerabilities between sexes, but this has yet to be directly investigated.

Thus, the aim of our experiments was to interrogate the effect of stress occurring during the specific pubertal window of each sex on anhedonia, anxiety, and antidepressant-like behavior in adolescence and determine whether these changes persist into adulthood. To this end, male and female Sprague Dawley rats were exposed to a three-day stress protocol during puberty (postnatal days 35–37 in females, 45–47 in males) (Harris et al., 2022; Horovitz et al., 2014) and underwent behavioral tests in either adolescence or adulthood. Monoamine neurotransmitter systems continue to develop throughout puberty and have been linked to depressive-like behavioral phenotypes in studies of antidepressant drugs (Cryan et al., 2005b; Forster et al., 2018; Pitzer, 2019) as well as reward and anhedonia, a core symptom of depression (Andersen and Teicher, 2008; Suri et al., 2015; Walker et al., 2017). Therefore, we additionally quantified the impact of pubertal stress on tissue concentrations of noradrenaline and serotonin in the brainstem and dopamine in the striatum in a separate cohort of adolescent and adult rats that had not undergone behavioral testing. Our hypothesis was that females stressed during puberty would be more vulnerable to develop depressive-like behavior compared to males and that pubertal stress would decrease brainstem noradrenaline concentrations and decrease turnover of brainstem serotonin and striatum dopamine, reflecting a similar female-biased vulnerability to stress.

2. Materials and methods

2.1. Animals

Male and female rats were bred in the Biological Services Unit at University College Cork from mating pairs of adult Sprague-Dawley rats obtained from Envigo (average litter size = 14 pups). Upon weaning at postnatal day (PND) 21, all offspring were housed in same-sex group housing under standard laboratory conditions (22 ± 2 °C, 12-h light/dark cycle with lights on at 07:00 a.m. and *ad libitum* access to food and water). Behavioral experiments took place during the light phase of the cycle (between 08:00 and 17:00). All experiments were conducted in accordance with international standards of animal welfare as outlined by European Directive 2010/63/EU and approved by the Animal Experimentation Ethics Committee of University College Cork. All experimenters had individual authorizations from the Health Products Regulatory Authority (HPRA) affiliated with HPRA Project Authorization AE19130/P084.

2.2. Pubertal stress paradigm

At PND 28, male and female rats with the same date of birth were randomly assigned to pubertal stress or control groups and housed in same-sex, same-treatment groups of 3–4 (litters mixed). No more than two littermates of the same sex were assigned to the same group. For cohort 1 of adolescent behavior, offspring were generated from 8 litters: control male n = 13, pubertal stress male n = 13, control female n = 15, pubertal stress female n = 15. For cohort 2 of adolescent behavior, offspring came from 7 litters: control male n = 9, pubertal stress male n = 9, control female n = 11, pubertal stress female n = 10. For adult behavior, offspring came from 8 litters: control male n = 13, pubertal stress male n = 13, control female n = 12, pubertal stress female n = 13.

Stressed rats underwent a daily stressor during the window of pubertal maturation in each sex: PND 35–37 in females, PND 45–47 in males (Lewis et al., 2002). Pubertal onset was confirmed by visual inspection of vaginal opening in females and preputial separation in males. The mean PND of vaginal opening in control females was 35.6 ± 1.65 and 36.1 ± 1.57 in stressed females. The mean PND of preputial separation in control males was 46.4 ± 1.98 and 46.0 ± 2.02 in stressed males. This stress paradigm was adapted from a 3-day pre-pubertal juvenile stress protocol established by Dr. Richter-Levin and colleagues (Harris et al., 2022; Horovitz et al., 2012). On the first day, rats were individually exposed to 10 min of swim stress in a cylindrical glass water tank (21-cm diameter) filled to 30-cm with water (22 ± 1 °C). On the second day, rats were placed on an elevated platform (platform dimensions: 12 × 12 cm, 70 cm above floor level) for three 30-min trials separated by 60-min inter-trial intervals in the home cage. On the third

day, rats were restrained in a clear acrylic rodent restrainer for 2 h. Control rats were left undisturbed during this period. Pubertal stress did not impact weight gain during the experiment. The rats then underwent behavioral testing either as adolescents (immediately following the stress) or as adults (beginning 4 weeks later; Fig. 1A). The adolescent cohort 1 underwent the saccharin preference test (SPT), open field test (OFT), blood withdrawal for corticosterone measurements, and the forced swim test (FST) [Fig. 1A]. Adolescent cohort 2 underwent the SPT, novelty-suppressed feeding test (NSF), elevated plus maze (EPM), and the FST. The adult cohort (Fig. 1A) underwent the female urine sniffing test (FUST) (males only), SPT, novelty-induced hypophagia (NIH), OFT, blood withdrawal for corticosterone measurements, and the FST.

To reduce the total number of rats required for the study, each rat underwent multiple behavioral tests. Rats tested in adulthood had 1–2 days of rest between behavioral tests and the more stressful tests (EPM, OFT, FST) were scheduled at the end of the behavioral battery to prevent acute stress-related impacts on future behavioral tests The window of adolescence is much shorter and therefore fewer behaviors were tested and the rats had fewer rest days.

A separate cohort of rats were euthanized in either adolescence (two days post-stress) or adulthood (6 weeks post-stress) without undergoing any behavioral tests. Brains were free-hand dissected to collect the whole striatum and brainstem, which were snap frozen on dry ice and stored at −80 °C until monoamine neurotransmitter analysis [Fig. 1B]. The brainstem was chosen as it contains nuclei that provide noradrenergic (locus coeruleus, LC) and serotonergic (dorsal and medial raphe nuclei) innervation of the brain. The striatum (encompassing both dorsal and ventral regions) was chosen due to its high concentration of dopamine and the associated role of the ventral striatum in hedonia and reward (Voorn et al., 2004). Adolescent control and pubertal stress rats that did not undergo behavior were littermates of the adolescent behavior-tested rats in cohort 2: control male n = 11, pubertal stress male n = 11, control female n = 12, pubertal stress female n = 12. Adult control and pubertal stress rats that did not undergo behavior were littermates of the adult behavior-tested rats: control male n = 11, pubertal stress male n = 11, control female n = 10, pubertal stress female n = 11.

2.3. Saccharin preference test (SPT)

The saccharin preference test is designed to quantify a loss of preference for a sweet, rewarding solution as a behavioral readout of anhedonia, the loss of pleasure in normally rewarding stimuli, which is a hallmark of depression in humans (Treadway and Zald, 2011). Adolescent and adult male and female rats were singly housed in a cage with two bottles for the two days of the test. Following one day of habituation to single housing and the presence of two water bottles in the cage, one of the bottles of water was replaced with a 0.01% saccharin solution (Sclafani et al., 2010). The volume of water and saccharin solution in both bottles was recorded every 12 h for 24 h. The positions of the two bottles were switched after each measurement to reduce any confound produced by a side bias. Saccharin preference was calculated as a percentage of the volume of saccharin intake over the total volume of fluid intake. The saccharin solution volume was corrected for each rat's body weight (mL/kg). After the last measurement, the rats were returned to their original group housing.

2.4. Female urine sniffing test (FUST)

As an additional measure of hedonic behavior in sexually mature adult males, we conducted the female urine sniffing test in adult males to test their preference for sexual stimulus as described previously (Levone et al., 2021a, 2021b; Malkesman et al., 2010; O'Leary et al., 2014). Rats were habituated to single housing in a small cage in a dimly lit testing room (~10 lux) for 45 min. During the last 20 min of this habituation, a cotton-tipped swab was inserted into the cage. In the test phase, the rat was exposed to a new cotton swab containing sterile water for 3 min. After a 45-min intertrial interval, the rat was exposed to new cotton swab infused with fresh urine collected from female rats in estrus for 3 min. The time spent sniffing and investigating the cotton swab in each 3-min trial was measured. A preference score was calculated as the time spent sniffing the urine divided by the total time spent sniffing both the urine and water-infused cotton swabs multiplied by 100 percent.

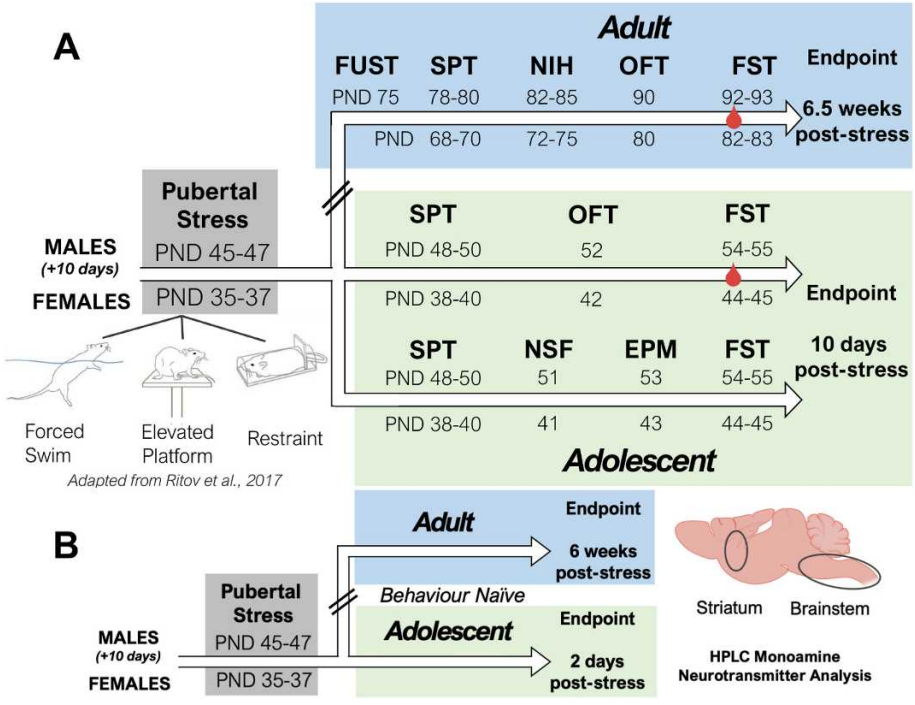


Fig. 1. Experimental timeline
A) **Behavioral study:** Timeline of procedures and behavioral tests performed in adulthood (blue panel) or adolescence (green panel).
B) **Behavior naïve brain tissue collection:** Brains from male and female rats were collected in adulthood or adolescence following pubertal stress. The striatum and brainstem were dissected and flash frozen and stored at −80 °C until monoamine neurotransmitter analysis.
Abbreviations: Elevated plus maze (EPM), forced swim test (FST), female urine sniffing test (FUST), high-performance liquid chromatography (HPLC), novelty-induced hypophagia (NIH), novelty-suppressed feeding (NSF), open field test (OFT), postnatal day (PND), saccharin preference test (SPT). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.5. Open field test (OFT)

The open field test is a widely used measure of locomotor activity and exploration (Prut and Belzung, 2003). After habituation to the testing room, adolescent and adult male and female rats were introduced to the brightly lit open field arena (90 cm diameter, 1100 lux) for 10 min. Rats were then removed and placed back into their home cage. The arena was cleaned between subjects with 70% ethanol to remove olfactory cues. Each test was video recorded, and the total distance traveled, time spent in the center 50% of the arena were scored using Ethovision XT 13. An observer blind to the experimental groups also scored rearing in the arena (when the animal briefly stands on hind limbs to explore an environment) (Sturman et al., 2018).

2.6. Novelty-induced hypophagia (NIH)

Novelty-induced hypophagia is a behavioral test of anxiety which measures hyponeophagia, the latency to approach palatable food in a novel environment, which has been adapted from a protocol previously used in mice (Dulawa and Hen, 2005; O'Leary et al., 2013). This test was only performed in adults because it requires several days of single housing, which would extend the behavioral testing window past the period of adolescence. All rats (adult males and females) were singly housed for the duration of the test. In the habituation phase, each animal was presented with a highly palatable solution of diluted sweetened condensed milk (1:4 in water) in their home cage for 30 min per day for 2 days. On days 3 and 4 (test days), the rats were brought to a testing room to habituate for 90 min prior to testing. On the test days, each animal was presented with the milk solution during a 10-min trial and their latency to drink the milk was measured. The test on day 3 was conducted in their home cage in the testing room under low illumination (~50 lux); whereas on day 4, the rats were in a novel, anxiogenic context [a new cage with no bedding and bright illumination (~1000 lux)]. Following the test on day 4, all rats were returned to their original group housing.

2.7. Novelty-suppressed feeding (NSF)

The novelty suppressed feeding test is based on a conflict between rodents' innate fear of brightly lit open spaces and motivation for food (Bodnoff et al., 1988). The latency to approach food in a novel, anxiogenic environment can be used as a measurement of anxiety-like behavior (Marcussen et al., 2008). All food was removed from the home cage approximately 18 h prior to testing. Male and female adolescent rats were habituated to the testing room for 45 min prior to the test. A single pellet of food was placed on a petri dish in the center of the brightly lit arena (90 cm diameter, 1100 lux) with approximately 3 cm of sawdust bedding on the floor. The walls of the arena were cleaned with 70% ethanol between rats to remove olfactory cues. The rat was introduced to the edge of the arena and allowed to explore for up to 10 min. The latency to begin eating the pellet was measured. As soon as the rat was observed to eat, or the 10-min time limit was reached, the rat was removed and placed in a single cage with a pre-weighed food pellet. Rats that had not eaten any of the pellet by the 10-min time limit were assigned a latency score of 600 s. The amount of food eaten in the home cage for the following 30 min was measured. Rats were returned to group housing after the test.

2.8. Elevated plus maze (EPM)

The elevated plus maze is a test widely used to assess anxiety-like behavior in rodents (Campos et al., 2013; Hogg, 1996). Following habituation to the dimly lit testing room (red lighting, 5 lux), adolescent male and female rats were introduced into the elevated plus maze and allowed to explore for 5 min. The maze consisted of 4 intersecting arms elevated 50 cm from the floor: 2 open arms (50 × 10 cm), 2 closed arms (50 × 10 cm with 40 cm walls), and a center area (10 × 10 cm). The test was video recorded from above and the time spent in each portion of the maze (open arms, closed arms, center) was scored using Ethovision XT 13. The percent of time spent in the open arms was calculated as the time in open arms divided by the total time in both closed and open arms multiplied by 100 percent.

2.9. Forced swim test (FST)

The forced swim test is a widely used model for assessing antidepressant-like behavior in rodents (Cryan et al., 2005b). Adolescent and adult male and female rats were individually placed in a glass tank (21-cm diameter) filled to 30-cm mark with water (24 ± 1 °C) (Porsolt et al., 1978). The forced swim test consisted of two sessions separated by 24 h: 15 min for the pre-swim (which was also used to measure the corticosterone response to stress) and 5 min for the test swim. Following both swims each rat was gently towel-dried then returned to its home cage. The 5-min test was recorded by a video camera. An observer blind to experimental groups used the time-sampling technique to score the predominant behavior (immobility, climbing, or swimming) for each 5-sec period of the 300-sec test. Passive (immobility) and active (climbing and swimming) behaviors were defined as described by Slattery and Cryan (2012) and developed by Detke et al. (1995). The observer also scored head shaking, defined as a rapid side-to-side movement of head above water, a behavior with a significant sex difference (Kokras et al., 2017). Males typically exhibit significantly more head shaking than females, a behavior which is decreased by antidepressant treatment in males (Kokras et al., 2018, 2017, 2015; Lino-De-Oliveira et al., 2005).

2.10. Plasma corticosterone measurements

The effects of pubertal stress on the corticosterone response to an acute stressor (pre-swim of the FST) was measured in both adolescent and adult male and female rats. For plasma corticosterone measurements, blood was collected immediately prior to the 15-min pre-swim (T0), 15 min after the pre-swim (T30), and 45 min after the pre-swim (T60). A sterile scalpel blade was used to make a small nick (<1 mm) at the tip of the tail. At each time point, approximately 120 μl of blood was collected from the tail tip into heparinized capillary tubes. The blood was centrifuged at 3500 RCF for 10 min. Plasma was collected and stored at −20 °C until analysis. Plasma corticosterone levels were assessed using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Enzo Life Sciences, ADI-900-097).

2.11. Monoamine neurotransmitter measurement using HPLC and electrochemical detection

The dissected striatum and brain stem tissue was sonicated in 1 mL of chilled mobile phase spiked with 2ng/20μl of N-Methyl 5-hydroxytryptamine (5-HT) (Sigma, UK) as internal standard. The mobile phase contained 0.1M citric acid, 0.1M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Cork, Ireland), 5.6 mM octane-1-sulphonic acid (Sigma) and 9% (v/v) methanol (Alkem/Reagecon) and was adjusted to pH 2.8 using 4N sodium hydroxide (Alkem/Reagecon). Homogenates were then centrifuged at 14,000 RPM for 20 min at 4 °C and 20 μL of the supernatant injected onto the HPLC system which was coupled to an electrochemical detector. A reverse-phase column (Kinetex 2.6u C18 100 × 4.6 mm, Phenomenex, UK) maintained at 30 °C was employed in the separation (Flow rate 0.9 ml/min). The glassy carbon working electrode combined with an Ag/AgCL reference electrode (Shimdazu) was operated at +0.8 V and the chromatograms generated were analyzed using Class-VP 5 software (Shimadzu). Each monoamine [noradrenaline, dopamine, 3,4-Dihydroxyphenylacetic acid (DOPAC), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA)] was identified by its characteristic retention time as determined by standard

injections which were run at regular intervals during the sample analysis. Analyte:Internal standard peak height ratios were measured and compared with standard injections to calculate the results. The quantified levels are reported as ng of metabolite per gram of brain tissue.

2.12. Statistical analysis

Using G*Power 3.1, we determined that the minimum sample size required to achieve an α of 0.05 and a power of 80% for behavioral experiments was n = 9 per group. Behavioral data, corticosterone measurements (individual time points and area under the curve), and neurotransmitter concentrations were analyzed by two-way ANOVA with significance set at α = 0.05 and followed by Fisher's LSD post hoc comparisons where appropriate. Three-way ANOVA with repeated measures was used to analyze plasma corticosterone across timepoints. Effect sizes are reported as partial eta squared values (η^2). All data are shown as mean +standard error of the mean. Statistical analysis and data visualization were performed using GraphPad Prism 8.

3. Results

3.1. Pubertal stress induced anhedonia in the saccharin preference test in adult males but not in females

Adolescent males had a higher saccharin preference [Main effect of sex: F (1, 50) = 5.86, p = 0.019, η2 = 0.10] [Fig. 2A] and consumed less saccharin solution per kg of body weight than adolescent females [Sex: F (1, 50) = 13.5, p = 0.0006, η^2 = 0.21] [Fig. 2B]. In adolescents, there were no significant effects of pubertal stress, nor an interaction between sex and pubertal stress on either saccharin preference or the volume of saccharin consumed per body weight (p > 0.05). In adults, there was an interaction effect of pubertal stress and sex [F (1, 45) = 6.58, p = 0.014, η^2 = 0.13] on saccharin preference [Fig. 2A]. Pubertal stress decreased saccharin preference in adult males (Fisher's LSD, p = 0.0057), but had no effect in adult females (p > 0.05). This resulted in a difference between the males and females of the pubertal stress group (p = 0.0005), but not the control group (p > 0.05). We found an interaction effect of pubertal stress and sex in the amount of saccharin solution consumed per body weight in adults [Stress × sex interaction: F (1, 45) = 6.53, p = 0.014, η^2 = 0.13] [Fig. 2B]. Pubertal stress males consumed less saccharin solution than control males (p = 0.0043) and, like the adolescents, control adult males consumed less saccharin per body weight than control adult females [Sex: F (1, 45) = 42.8, p < 0.0001, η^2 = 0.49].

In the female urine sniffing test (FUST), a test of anhedonia measuring sexual interest in adult males (Malkesman et al., 2010), pubertally stressed adult males performed similarly to control males. The preference for sniffing the female urine-soaked cotton bud was not different between groups [t (24) = 0.47, p = 0.124, η^2 = 0.096] [Fig. 2C].

3.2. Sex-dependent effects of pubertal stress on striatal dopamine turnover

To determine whether the anhedonic effect of pubertal stress in the saccharin preference test in adult males was associated with altered dopamine neurotransmission, we measured tissue concentrations of dopamine and its metabolite, DOPAC, in the striatum using HPLC. In both adolescent and adult striatum, we found an interaction effect of pubertal stress and sex on dopamine turnover (DOPAC/Dopamine) [Adolescent: F (1, 42) = 8.89, p = 0.005, η^2 = 0.12; Adult: F (1, 38) = 21.6, p < 0.001, η^2 = 0.45] [Fig. 2D]. There was an opposing effect of pubertal stress in males and females, where pubertal stress decreased

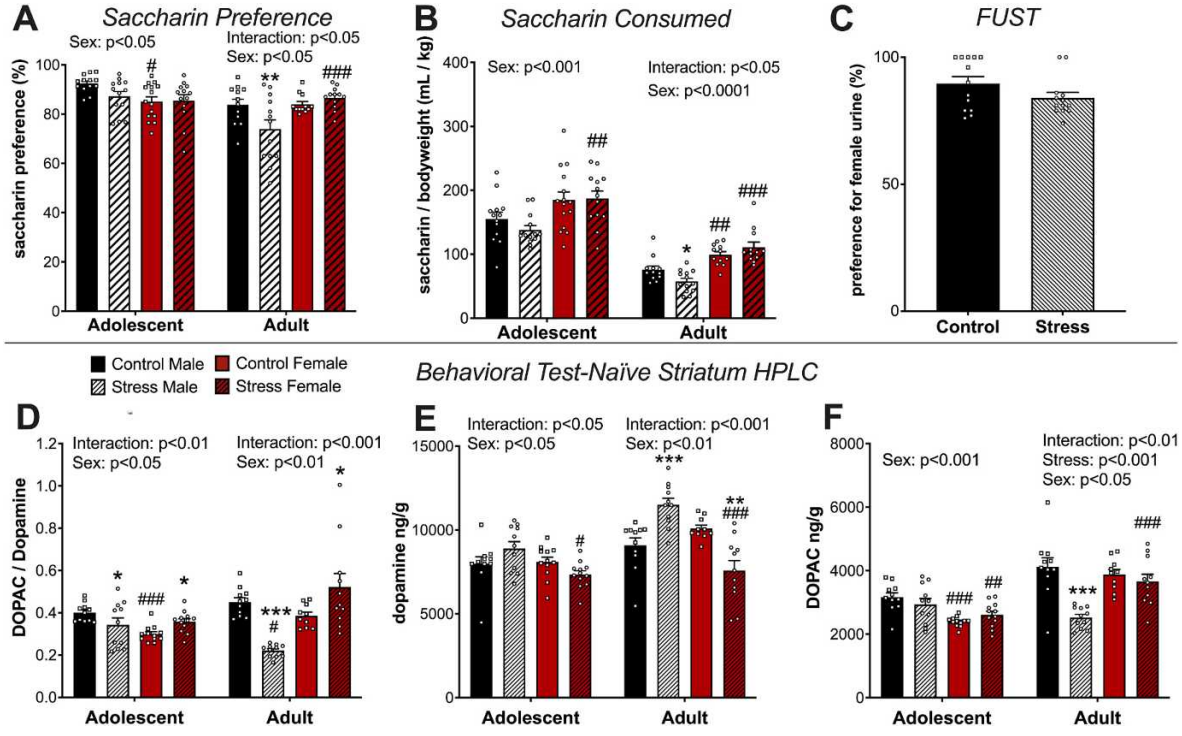


Fig. 2. Pubertal stress decreased saccharin preference in males and altered striatum dopamine turnover in a sex-specific manner. A) Saccharin preference, B) Milliliters of saccharin consumed per kg body weight, C) Adult male preference for female urine in the FUST, D) Behavioral test-naïve striatum dopamine turnover E) Striatum dopamine (ng/g tissue), F) Striatum DOPAC (ng/g tissue)
Two-way ANOVA effects listed above each measurement.
Significantly different to non-stress group of same sex: *p < 0.05, **p < 0.01, ***p < 0.001; Fisher's test.
Significantly different to corresponding group of opposite sex: #p < 0.05, ##p < 0.01, ###p < 0.001; Fisher's test.
SPT: n = 13–15 adolescents, n = 11–13 adults; FUST: n = 13 adult males; Behavioral test-naïve striatum: n = 11–12 adolescent; n = 10–11 adult.

dopamine turnover in adolescent and adult males [Adolescent: $p = 0.045$; Adult: $p = 0.0002$] and increased turnover in adolescent and adult females [Adolescent: $p = 0.037$; Adult: $p = 0.016$] compared to same sex controls. We also found an effect of sex on dopamine turnover in both adolescents and adults [Adolescent main effect of sex: $F(1, 42) = 4.99$, $p = 0.031$, $\eta^2 = 0.11$; Adult: $F(1, 38) = 10.7$, $p = 0.0022$, $\eta^2 = 0.22$] where control adolescent males had greater dopamine turnover than control adolescent females ($p = 0.0006$) and stressed adult females had greater turnover than stressed adult males ($p < 0.001$) [Fig. 2D].

In adolescents, the effect of pubertal stress on dopamine turnover arose from alterations in dopamine (Fig. 2E) concentrations [Pubertal stress × Sex interaction: $F(1, 42) = 5.97$, $p = 0.019$, $\eta^2 = 0.12$], rather than its metabolite, DOPAC ($p > 0.05$, Fig. 2F). However, DOPAC was higher in males compared to females [Sex: $F(1, 42) = 18.2$, $p = 0.0001$, $\eta^2 = 0.30$; Fig. 2F]. In adults, we found an interaction between pubertal stress and sex on both dopamine concentrations [$F(1, 39) = 31.8$, $p < 0.0001$, $\eta^2 = 0.45$] and DOPAC concentrations [$F(1, 39) = 10.6$, $p = 0.0023$, $\eta^2 = 0.21$]. Specifically, pubertal stress increased dopamine in adult males compared to control ($p = 0.0003$) and decreased dopamine in adult females compared to control ($p = 0.0003$). Pubertal stress decreased DOPAC only in adult males ($p < 0.0001$), whereas adult females were unaffected ($p > 0.05$) [Fig. 2F].

3.3. Pubertal stress decreased exploratory rearing specifically in adult females in the open field test and had no effect on anxiety-like behavior in either sex in adolescence or adulthood

Pubertal stress did not impact locomotor activity in the open field in adolescents [Stress: $F(1, 50) = 0.002$, $p > 0.05$, $\eta^2 = 0.05$], but there was an interaction of stress and sex in adults [$F(1, 46) = 5.94$, $p = 0.019$, $\eta^2 = 0.11$; Fig. 3A]. While there was no effect of pubertal stress on rearing in adolescents in either sex, stress impacted rearing behavior in adults [Stress: $F(1, 45) = 6.61$, $p = 0.014$, $\eta^2 = 0.13$] [Fig. 3B]. Fisher's LSD test revealed that pubertal stress decreased rearing specifically in adult females ($p = 0.0065$) but not in males ($p > 0.05$).

Females were more active and exploratory than males in both adolescence and adulthood. Females exhibited increased locomotor activity compared to males, as measured by the total distance traveled in the arena [Effect of sex in adolescents: $F(1, 50) = 7.50$, $p = 0.0085$, $\eta^2 = 0.13$; Effect of sex in adults: $F(1, 46) = 9.02$, $p = 0.004$, $\eta^2 = 0.16$] [Fig. 3A]. Females also exhibited more exploratory rearing behaviors than males in both adolescence [Sex: $F(1, 50) = 4.39$, $p = 0.041$, $\eta^2 = 0.08$] and adulthood [Sex: $F(1, 45) = 16.8$, $p = 0.0002$, $\eta^2 = 0.27$] [Fig. 3B]. There was no effect of pubertal stress, sex, or their interaction on the time spent in the center of the arena, a measure of anxiety-like behavior, in adolescents and adults ($p > 0.05$) [Fig. 3C].

To further determine the impact of pubertal stress on anxiety-like behavior, adolescents were tested in the elevated plus maze [Fig. 3D] and novelty-suppressed feeding test [Fig. 3E]. In the EPM, we found no effect of pubertal stress, sex, or their interaction on the percent of time spent in the open arms ($p > 0.05$) [Fig. 3D]. Likewise, in the NSF test, pubertal stress did not affect the latency to eat in the center of the arena ($p > 0.05$). There was no effect of sex or an interaction between stress and sex ($p > 0.05$) [Fig. 3E]. Adults were assessed for anxiety-like behavior in the novelty-induced hypophagia test, where we found no effect of pubertal stress, sex, or their interaction on the net latency to drink the palatable milk solution in the novel cage ($p > 0.05$) [Fig. 3F].

3.4. Pubertal stress prolonged corticosterone response to acute stress in adolescent females but not adults

To investigate if prior pubertal stress alters the corticosterone

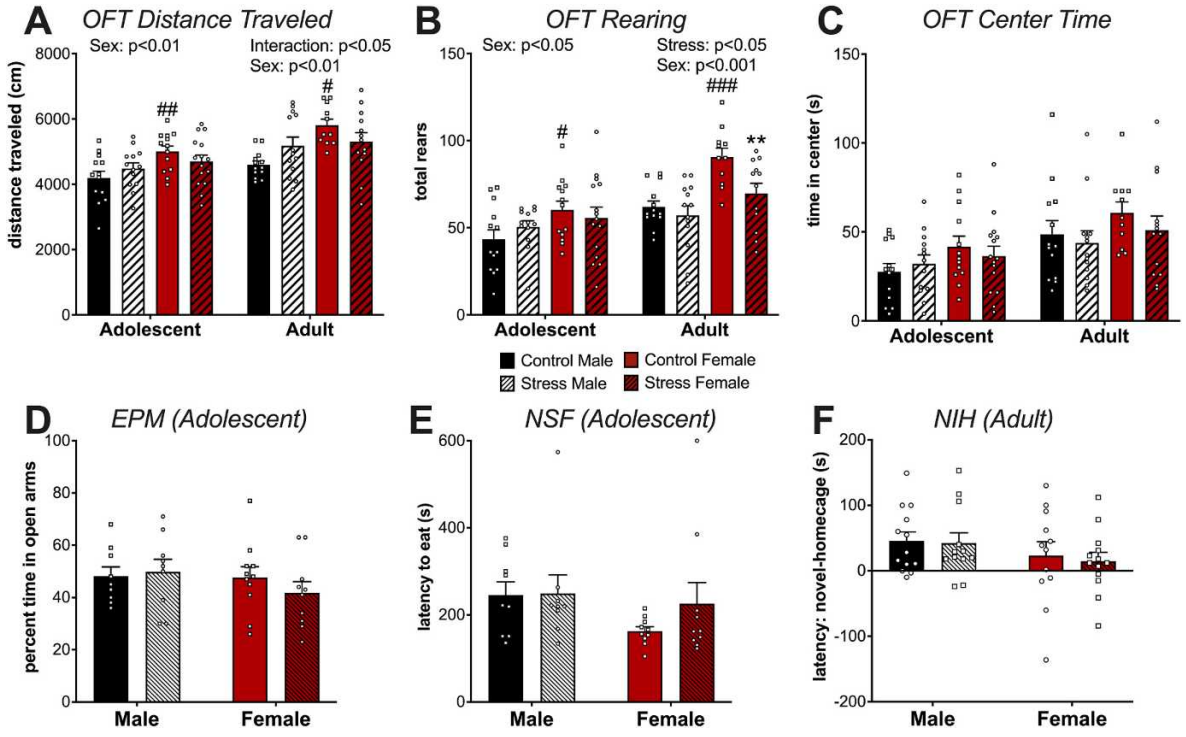


Fig. 3. Effects of pubertal stress on locomotor activity, exploration, and anxiety-like behavior. A) Total distance traveled in open field (cm), B) total number of rearing behaviors, C) time in center of the open field arena, D) percent of time spent in open arms in EPM in adolescents, E) latency to eat in NSF test in adolescents, F) net latency to drink palatable solution in NIH test in adults

Two-way ANOVA effects listed above each measurement.

Significantly different to non-stress group of same sex: **$p < 0.01$ Fisher's test; Significantly different to corresponding experimental group of opposite sex: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, Fisher's test

OFT: n = 13–15 adolescents, n = 11–13 adults; EPM: n = 9–11 adolescents; NSF: n = 8–10 adolescents; NIH: n = 12–13 adults.

response to acute stress later in either adolescence or adulthood, plasma corticosterone was measured prior to and after the 15-min pre-swim of the FST. Plasma corticosterone was quantified at three time points: immediately prior to the 15-min pre-swim (T0), 15 min after the swim (T30), and 45 min after the swim (T60). In both adolescents and adults, plasma corticosterone concentrations significantly differed across time [Adolescent main effect of time: $F(2, 56) = 376.1$, $p < 0.0001$, $\eta^2 = 0.93$; Adult main effect of time: $F(2, 51) = 523.3$, $p < 0.0001$, $\eta^2 = 0.95$] [Fig. 4A and F]. There was also an interaction between sex and time in both adolescents and adults [Adolescent: $F(2, 56) = 3.28$, $p = 0.045$, $\eta^2 = 0.10$; Adult: $F(2, 51) = 25.8$, $p < 0.0001$, $\eta^2 = 0.45$]. Adult females had higher plasma corticosterone concentrations compared to adult males [Effect of sex: [$F(1, 28) = 21.7$, $p < 0.0001$, $\eta^2 = 0.54$] [Fig. 4E]. Pubertal stress increased plasma corticosterone only in adolescents [Stress: $F(1, 28) = 5.12$, $p = 0.031$, $\eta^2 = 0.18$] and not in adults ($p > 0.05$) [Fig. 4A and F]. There was no effect of sex, pubertal stress, or their interaction on area under the curve (AUC) for adolescents [$p > 0.05$; Fig. 4B]. In adults, AUC was greater in females compared to males [Sex: $F(1, 79) = 9.81$, $p = 0.0024$, $\eta^2 = 0.11$; Fig. 4G], but there was no effect of pubertal stress or an interaction of sex and stress ($p > 0.05$). Given the strong effect of time on corticosterone, each time point was considered separately within adolescents and adults.

Female adolescents had higher basal (T0) corticosterone concentrations compared to male adolescents [Sex: $F(1, 28) = 5.38$, $p = 0.028$, $\eta^2 = 0.16$] [Fig. 4C]. At T30, there were no effects of stress, sex, or their interaction ($p > 0.05$) [Fig. 4D]. At T60, plasma corticosterone of stressed rats remained elevated compared to control [Stress: $F(1, 28) = 8.41$, $p = 0.0072$, $\eta^2 = 0.23$] [Fig. 4E]. Pairwise comparisons revealed that stressed females had higher corticosterone than female controls at T60 ($p = 0.026$, Fisher's test), but there was no difference between pubertally stressed males and their controls at T60 ($p > 0.05$). In adults, females had higher plasma corticosterone concentrations compared to male adults at T30 [Sex: $F(1, 28) = 31.0$, $p < 0.0001$, $\eta^2 = 0.53$; Fig. 4I] and T60 [Sex: $F(1, 28) = 13.9$, $p = 0.0009$, $\eta^2 = 0.33$; 4J], but this did not reach statistical significance at T0 [Sex: $F(1, 23) = 3.46$, $p = 0.075$, $\eta^2 = 0.13$]. Contrary to findings in adolescence, pubertal stress did not affect plasma corticosterone at any time point in either male of female adults ($p > 0.05$).

3.5. *Pubertal stress increases immobility in forced swim test and exerts sex-specific effects on escape-oriented behaviors in adolescence but not adulthood*

Pubertal stress increased immobility in the forced swim test in both adolescent [Stress: $F(1, 52) = 14.1$, $p = 0.0004$, $\eta^2 = 0.21$] and adult [Stress: $F(1, 45) = 13.8$, $p = 0.0006$, $\eta^2 = 0.23$] male and female rats [Fig. 5A and B]. This effect was manifested via different behavioral strategies in a sex-dependent manner in adolescents but not adulthood. In adolescents, we found an interaction of pubertal stress and sex in climbing behavior [Stress × Sex interaction: $F(1, 52) = 6.8$, $p = 0.012$, $\eta^2 = 0.12$], whereby pubertal stress decreased climbing in adolescent males ($p = 0.011$) but not adolescent females ($p > 0.05$). We also found an interaction effect for swimming behavior [Stress × Sex interaction: $F(1, 52) = 5.2$, $p = 0.026$, $\eta^2 = 0.09$], where pubertal stress decreased swimming behaviors specifically in adolescent females ($p < 0.0001$) but not in adolescent males ($p > 0.05$) [Fig. 5A]. However, in adults,

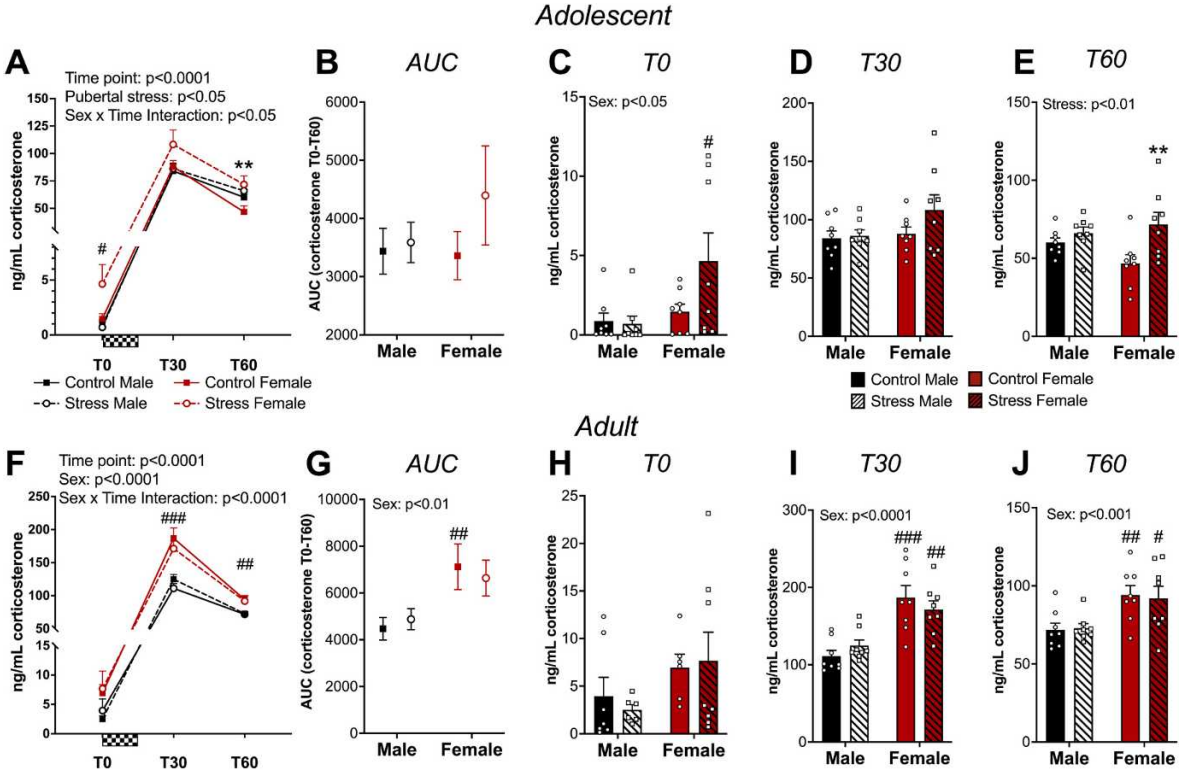


Fig. 4. Plasma corticosterone levels following acute stress in adolescents and adults

A) Time course of plasma corticosterone in adolescents, B) area under the curve (AUC), C) T0, D) T30, and E) T60 in adolescents; F) Time course of plasma corticosterone in adults, G) area under the curve (AUC), H) T0, I) T30, J) T60 in adults.

Checked rectangle at T0 indicates the acute stressor (15-min pre-swim)

Three-way ANOVA effects listed above A and E. Two-way ANOVA effects listed above B, C, D, F, G, and H. Symbols indicate significant Fisher's test in Two-Way ANOVA.

Significantly different to non-stress group of same sex: **$p < 0.01$ Fisher's test; Significantly different to corresponding group of opposite sex: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, Fisher's test

n = 8 adolescents, n = 6–8 adults.

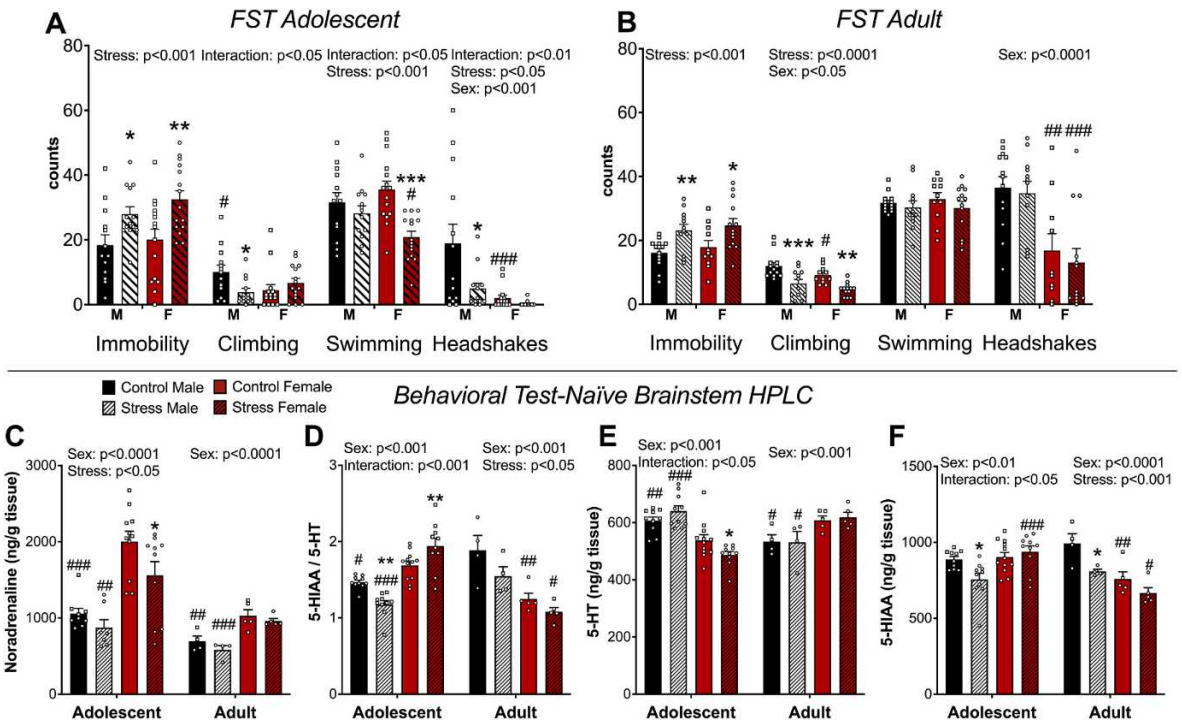


Fig. 5. Pubertal stress increases immobility in forced swim test and exerts sex-specific effects on escape-oriented behaviors and brainstem noradrenaline concentrations and 5-HT turnover

Counts of immobility, climbing, swimming, and headshake behaviors during the 5-min forced swim test in A) adolescents and B) adults (C) Behavioral test-naïve brainstem noradrenaline (ng/g) (D) Brainstem serotonin turnover 5-HIAA/5-HT (E) 5-HT (ng/g tissue) (F) 5-HIAA (ng/g tissue).

Two-way ANOVA effects listed above each measurement. FST: n = 13–15 adolescents, n = 11–13 adults. Behavioral test-naïve brainstem: n = 10–12 adolescent; n = 4–5 adult

Significantly different to non-stress group of same sex: *p < 0.05, **p < 0.01, ***p < 0.001, Fisher's test

Significantly different to corresponding group of opposite sex: #p < 0.05, ##p < 0.01, ###p < 0.001, Fisher's test.

pubertal stress decreased climbing in both sexes [Stress: $F(1, 45) = 26.9$, $p < 0.0001$, $\eta^2 = 0.37$] while swimming was unaffected in both sexes ($p > 0.05$) [Fig. 5B]. Sex impacted FST behaviors in both adolescence and adulthood. Adolescent and adult females engaged in fewer climbing behaviors compared to their male counterparts [Adolescent: control male versus control female, $p = 0.018$ (Fisher's LSD); Adult effect of sex: $F(1, 45) = 5.76$, $p = 0.021$, $\eta^2 = 0.11$].

Pubertal stress also impacted head shake frequency, a behavior with a robust sex difference where males exhibit more head shakes than females (Kokras et al., 2017). In adolescents, pubertal stress reduced head shakes in males compared to controls [Stress × sex interaction: $F(1, 52) = 5.2$, $p < 0.05$, $\eta^2 = 0.08$] but not in adults ($p > 0.05$). As expected, headshakes were also significantly higher in males compared to females at both ages [Adolescent effect of sex: $F(1, 52) = 13.75$, $p = 0.0005$, $\eta^2 = 0.21$; Adult effect of sex: $F(1, 45) = 25.81$, $p < 0.0001$, $\eta^2 = 0.36$] [Fig. 5A and B].

3.6. Pubertal stress induces sex-specific effects on brainstem serotonin turnover and noradrenaline concentrations in adolescence but not adulthood

Previous preclinical studies of antidepressant drugs have pointed to connections between monoamine neurotransmitters and specific behaviors in the FST. In particular, climbing behavior has been linked to noradrenergic signaling (Cryan et al., 2002, 2005a; Kelliher et al., 2003), whereas swimming behavior has been linked to serotonergic neurotransmission (Cryan et al., 2005a; Detke et al., 1995; Page et al., 1999). Thus, in a separate cohort of rats that had not undergone behavioral tests, we investigated whether pubertal stress altered 5-HT turnover and noradrenaline in the brainstem in a sex-specific manner [Fig. 3 C–F].

Pubertal stress decreased noradrenaline concentrations in adolescent brainstem [Stress: $F(1, 33) = 5.06$, $p = 0.031$, $\eta^2 = 0.12$]. Post hoc tests revealed that the effect of pubertal stress was significant in adolescent females ($p = 0.036$) but not in adolescent males ($p > 0.05$). There was no effect of pubertal stress on noradrenaline in adult brainstem ($p > 0.05$) in either sex [Fig. 5C]. Females had higher brainstem noradrenaline levels than males in both adolescence and adulthood [Sex effect in adolescents: $F(1, 33) = 34.3$, $p < 0.0001$, $\eta^2 = 0.51$; Sex effect in adults: $F(1, 14) = 33.6$, $p < 0.0001$, $\eta^2 = 0.71$] [Fig. 5C].

Serotonin (5-HT) turnover, as measured by 5-HIAA/5-HT, was affected by pubertal stress in a sex-specific manner in adolescents [Stress × sex interaction: $F(1, 38) = 20.1$, $p < 0.0001$, $\eta^2 = 0.32$], whereby stress decreased turnover in males ($p = 0.0034$) and increased turnover in females ($p = 0.0062$) [Fig. 5D]. In adult brainstem, there was an overall effect of pubertal stress on 5-HT turnover [Stress: $F(1, 14) = 5.3$, $p < 0.05$, $\eta^2 = 0.26$]. The effect of pubertal stress on 5-HT turnover in adolescent brainstem was due to sex-specific changes in 5-HT [Stress × sex interaction: $F(1, 38) = 6.74$, $p = 0.013$, $\eta^2 = 0.15$; Fig. 5E] and 5-HIAA [Stress × sex interaction: $F(1, 38) = 6.53$, $p = 0.015$, $\eta^2 = 0.15$; Fig. 5F]. Pubertal stress decreased 5-HT in adolescent females ($p = 0.029$) compared to controls but not adolescent males [Fig. 5E], whereas stress decreased 5-HIAA in brainstem of adolescent males compared to controls ($p = 0.0083$) but not adolescent females [Fig. 6F]. The effect of pubertal stress on 5-HT turnover in adult brainstem was due to an overall stress effect on 5-HIAA [Stress: $F(1, 14) = 9.82$, $p = 0.007$, $\eta^2 = 0.41$], but not 5-HT ($p > 0.05$) [Fig. 5E and F].

There was an age-specific effect of sex on 5-HT turnover as well as the individual metabolites at both ages. [Sex: $F(1, 38) = 59.8$, $p < 0.0001$, $\eta^2 = 0.61$] and adults [Sex: $F(1, 14) = 23.6$, $p = 0.0003$, $\eta^2 = 0.63$]. In adolescents, 5-HT turnover was higher in females, but in adults, 5-HT turnover was higher in males [Fig. 5D]. For 5-HT, males

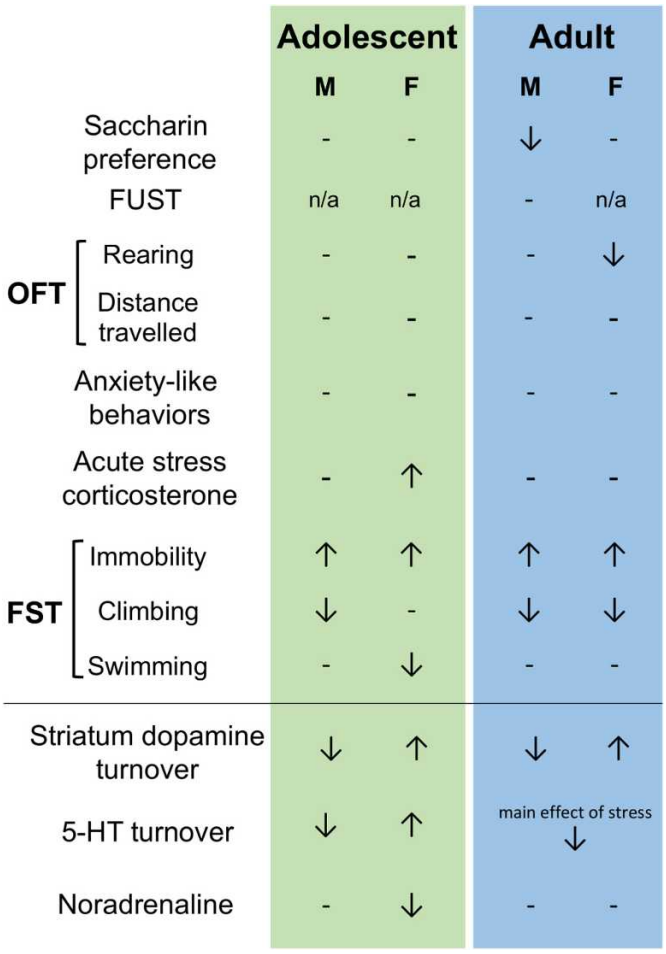


Fig. 6. Summary of effects of pubertal stress on behavior, corticosterone, and neurotransmitters in males and females in adolescence and adulthood
Arrows indicate direction of pubertal stress effect relative to same-sex controls. - indicates no significant difference from control. M = Male, F = Female.

had higher concentrations in adolescence compared to females [Sex: F (1, 38) = 45.3, $p < 0.0001$, $\eta^2 = 0.54$], whereas females had higher concentrations in adulthood compared to males [Sex: F (1, 14) = 12.1, p = 0.004, $\eta^2 = 0.46$]. 5-HIAA concentrations followed a similar pattern where adolescent females had higher 5-HIAA concentrations than adolescent males [Sex: F (1, 38) = 9.1, p = 0.005, $\eta^2 = 0.19$] and adult males had higher concentrations than adult females [Sex: F (1, 14) = 18.3, p = 0.0008, $\eta^2 = 0.41$].

4. Discussion

Evidence from clinical studies has shown that women are more susceptible to develop stress-related disorders like major depression and PTSD (Kessler et al., 1993; Kuehner, 2017; Seedat et al., 2009). The symptom profiles of depressive disorders also manifest differently in men and women. For example, women report more atypical symptoms than men (E.g. increased appetite and hypersomnia), whereas men show a higher comorbidity of substance use disorders (Marcus et al., 2008). The female bias in prevalence of stress-related disorders emerges at puberty (Gutiérrez-Lobos et al., 2002; Kessler et al., 1993; Kwong et al., 2019), which suggests that puberty may be an important critical window during which time stress may disproportionately impact neurobehavioral outcomes although this not been widely investigated. Therefore, our study used a 3-day stress paradigm applied specifically during the time of pubertal onset in males and females to assess the short- and long-term impacts on behavior and neurotransmitter concentrations.

We found that short-term pubertal stress had sex-specific effects on behavior in adolescence and adulthood (summarized in Fig. 6). We also found sex-specific effects of this stressor on concentrations of certain neurotransmitters in adolescent and adult rats that did not undergo behavioral tests (summarized in Fig. 6). Female-specific effects of pubertal stress included decreased exploratory rearing behavior in adults in the open field, prolonged corticosterone response to an acute stressor in adolescents, reduced swimming behavior in the FST in adolescents, increased brainstem serotonin turnover, decreased brainstem noradrenaline concentrations in adolescents, and increased striatal dopamine turnover at both ages. Other pubertal stress effects were specific to males, including increased anhedonia in the SPT in adults, decreased climbing behavior and headshakes in adolescents, decreased striatal dopamine turnover in adolescents and adults, and decreased brainstem serotonin turnover in behavioral test-naïve adolescents. While pubertal stress increased immobility in the FST in both sexes in both adolescence and adulthood, pubertal stress altered escape-oriented behaviors of adolescents in a sex-specific manner, whereby in adolescence, prior pubertal stress decreased climbing behavior in adolescent males only and decreased swimming behavior in adolescent females only. In behavioral test-naïve adolescents, pubertal stress had opposing effects in males and females on adolescent brainstem serotonin turnover, a neurotransmitter implicated in swimming behavior in the FST (Cryan et al., 2005a; Detke et al., 1995; Page et al., 1999).

4.1. Pubertal stress increased anhedonia specifically in adult males

A hallmark symptom of depression in humans is anhedonia, the loss of pleasure in normally rewarding stimuli (Treadway and Zald, 2011). Anhedonia can be quantified in rodents by measuring their preference for rewarding, sweet solutions (saccharin or sucrose) over regular water. We found that short-term pubertal stress induced anhedonia in the saccharin preference test specifically in adult males, but not females.

Dopamine is important for processing rewarding stimuli and growing evidence points to dopaminergic deficits in major depression (Dunlop and Nemeroff, 2007; Pizzagalli, 2014). In adult rats and mice, treatment with dopamine enhancing antidepressant drugs can attenuate stress-induced anhedonia (Harkin et al., 2002; Papp et al., 1993; Willner et al., 1994). Additionally, the dopaminergic system continues to develop during adolescence (Andersen et al., 2000; Chisholm et al., 2013; Juraska and Willing, 2017; Naneix et al., 2012) and may therefore be particularly susceptible to disruption by stressors. In male rats, dopamine receptors D1 and D2 expression in the mPFC and striatum peak during adolescence, due to an overproduction of receptors that are gradually pruned to adult levels (Andersen et al., 2000, 2002; Teicher et al., 1995). Dopamine innervation of the mPFC steadily increases during adolescence (Naneix et al., 2012; Willing et al., 2017). Previous research has also shown that there are sex differences in the striatum during early adolescence, where females have less dopamine receptor overproduction and less pruning compared to males (Andersen et al., 1997). Given the restructuring of the dopamine system during development and its important role in reward processing, we sought to determine if pubertal stress altered dopamine turnover in adolescents and adults by measuring dopamine and its metabolite DOPAC in the striatum in a separate cohort of control and pubertal stress rats that had not undergone behavioral testing. We hypothesized that pubertal stress would have a sex-specific effect on dopamine turnover, where males would be more affected by pubertal stress, perhaps more strongly in adults compared to adolescents given the behavioral results.

In line with our findings in the SPT, dopamine turnover (DOPAC/dopamine) in the striatum of behavioral test-naïve rats was decreased in adult males exposed to pubertal stress, and to a lesser extent in adolescent males. In adult males, both dopamine and DOPAC were affected by pubertal stress, perhaps indicating alterations in both dopamine synthesis and catabolism in the striatum. Although an increase in dopamine

concentrations may appear to conflict with increased anhedonia behavior, the decreased DOPAC concentration in pubertal stress adult males (resulting in decreased dopamine turnover) emphasizes the importance of measuring turnover as an indicator of dopamine function rather than dopamine alone.

Pubertal stress also increased striatal dopamine turnover in adolescent and adult females, which was driven by decreased dopamine concentrations without affecting DOPAC concentrations. This was unexpected, since pubertal stress did not affect saccharin preference behavior in adolescent or adult females. This sex-specific response to stress during pubertal maturation may indicate a compensatory mechanism in striatal dopamine concentrations in stressed females, where an increase in dopamine turnover prevented an anhedonic effect of pubertal stress. We also found sex differences in dopamine turnover in control males and females, where males had higher striatal dopamine turnover than females. This is in line with previous research establishing that basal dopamine tone in the adult striatum is higher in males than in females (Becker, 1999; Xiao and Becker, 1994). Females also exhibit less dopamine receptor overproduction during adolescence in the striatum compared to males, particularly in the dorsal striatum (Andersen et al., 1997). It has been previously reported that this sex difference in the overproduction of dopamine receptors in the adolescent striatum is not dependent on surges of gonadal hormones during puberty (Andersen et al., 2002). However, ovarian hormones do contribute to significant sex differences in dopamine activity in the striatum and can regulate dopamine-mediated motivated behaviors in adult females (Yoest et al., 2018).

To the best of our knowledge, this is the first time a short-term stressor applied specifically during sex-specific pubertal maturation periods has been reported to disrupt the dopamine system. We have previously shown that the same short-term 3-day stress paradigm during the pre-pubertal juvenile period (PND 27–29) increased immunoreactivity of tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis, in the medial prefrontal cortex in both males and females (Harris et al., 2022). Longer durations of adolescent stress have been shown to affect dopaminergic neurotransmission. For example, peri-pubertal stress (PND 28–42) in male rats increased expression of monoamine oxidase A in the prefrontal cortex (PFC) (Márquez et al., 2013). Adolescent social defeat in male rats (PND 35–40) has been shown to increase D1 receptor binding in the striatum in adulthood (Novick et al., 2011). These studies support our finding that short-term stress during puberty in males induces long-lasting changes in striatum dopamine turnover in behavioral test-naïve rats. Future research should address the impact of pubertal stress on other aspects of dopaminergic system, such as synapse pruning, receptor expression in other important brain regions related to anhedonia, such as the prefrontal cortex.

In contrast to the SPT, we found no effect of pubertal stress on adult males in the female urine sniffing test, another test of hedonic behavior (Malkesman et al., 2010). This may be because FUST and SPT measure responses to different modalities of rewarding stimuli: preference for sweet substances versus the drive to investigate sexually relevant stimuli. While some previous studies have shown that chronic variable stress in adult male mice can decrease preference for female urine in the FUST (Finger et al., 2011; Wang et al., 2021), no studies have investigated the long-term effects of adolescent stress in the FUST. This might indicate that this sexually motivated hedonic behavior is resistant to disruption by pubertal stress.

4.2. Pubertal stress did not impact anxiety-like behavior

In rats and mice, stress during adolescence (for a duration of between 10 days to several weeks) has been shown to increase certain anxiety-like behaviors in adulthood in the EPM and OFT (reviewed in McCormick and Green, 2013). However, our short-term stress paradigm applied specifically during the puberty stage of adolescence did not affect the time spent in the anxiogenic center of the open field arena in either sex in adolescence or adulthood. In adolescents, two further tests of anxiety-like behavior (EPM and NSF) revealed no effect of pubertal stress in either males or females. In agreement with our results, one study found that three days of stress from PND 44–46 in male Wistar rats did not affect anxiety-like behavior in the OFT (females were not studied) (Zalsman et al., 2015). Likewise, in adulthood, we found no effect of pubertal stress on anxiety-like behavior in either sex in the novelty-induced hypophagia test. These findings may suggest that either the pubertal stage of adolescence is not sensitive to stress-induced anxiety or that a three-day stress paradigm may not be sufficiently intense or long enough to impact anxiety-like behavior.

The OFT is also used to quantify general locomotor and exploratory activity. In adolescents and adults, females were more active and exploratory as measured by distance traveled and rearing behaviors, a sex difference that is well-documented in the literature (Alonso et al., 1991; Kokras et al., 2012; Lovelock and Deak, 2019; Slob et al., 1981). Additionally, we found a female-specific decrease in exploratory rearing behaviors in adult females exposed to stress during puberty. This is in line with a previous report from our lab, which found that short-term stress in the pre-pubertal juvenile period also decreased exploratory rearing in adult females but not males in the OFT (Harris et al., 2022). Predator odor stress during adolescence (5 exposures between PND 40–48) decreased rearing in the OFT in adult males and females (although sex was not analyzed as a variable) (Wright et al., 2008, 2013). This suggests that the stress effect on exploratory rearing in adult females may not be restricted to a specific period of development.

4.3. Pubertal stress prolonged the acute stress response in adolescent females

Dysregulation of the neuroendocrine stress response is thought to precede the development of psychiatric disorders like PTSD and depression (Lupien et al., 2009). The HPA axis undergoes significant maturation during puberty and adolescence which suggests that disruption of the stress response may underlie some behavioral effects of pubertal stress (Green and McCormick, 2016; Klein and Romeo, 2013). We found females that underwent short-term stress during puberty had a prolonged corticosterone response to a 15-min forced swim in adolescence compared to female controls, but there was no effect of pubertal stress on corticosterone concentrations in adolescent males. Although pubertal stress resulted in higher corticosterone at T60 (i.e., 45 min post FST stress) in adolescent females compared to control females, stressed adolescent females also tended to have higher baseline corticosterone, potentially indicating a flattening of the stress response curve. However, additional time points would be needed to determine whether this response extends beyond 45 min post-stress. This effect of pubertal stress on corticosterone did not persist into adulthood.

We found a sex difference in corticosterone concentrations at baseline (T0), where adolescent females had higher corticosterone compared to adolescent males. Similarly, adult females had greater corticosterone response to acute stress compared to adult males as measured by the area under the curve, a sex difference which is well described in the literature (reviewed in Bangasser and Valentino, 2014). Overall, these data indicate that pubertal stress leads to a short-lived disruption of the acute stress response, particularly in females, but not a long-lasting change in HPA axis responsivity. In agreement with our results, previous studies have shown that 15 days of chronic social stress in rats during an adolescent window that also included both male and female pubertal windows (PND 30–45) increased corticosterone response 45 min after an acute swim stress in adolescent stressed females compared to control females (Mathews et al., 2008). However, adolescent chronic social stress also enhanced the acute corticosterone stress response in adolescent males (Mathews et al., 2008). Similar to the current study, the effect of chronic social stress on the acute stress response did not persist into adulthood (Mathews et al., 2008; McCormick et al., 2005, 2008), suggesting that stressors during adolescence may have

shorter-term impacts on corticosterone reactivity to acute stress.

4.4. Pubertal stress impacts FST behavior and brainstem monoamine neurotransmitters

We found that short-term stress occurring during the pubertal window increased immobility in the FST in both males and females in adolescence and adulthood. In adolescence, pubertal stress had a sex-specific effect on escape-oriented behaviors in the FST. Pubertal stress decreased climbing in male adolescents only and decreased swimming behavior only in female adolescents. While the increased immobility persisted into adulthood, pubertal stress decreased climbing in both adult females and males with no effect on swimming behavior. Previous research has indicated that stress during adolescence can affect both active and passive coping behaviors in the FST. For example, chronic adolescent stress (Mixed modality stressors, PND 37–49) in Wistar rats resulted in female-specific decreased latency to float in FST and decreased "struggling" (analogous to climbing) in both adolescence and adulthood. However, chronic adolescent stress did not elicit any changes in FST behaviors in males in either adolescence or adulthood (Bourke and Neigh, 2011). Peripubertal stress (variable stressors on 7 days between PND 28–42) in Wistar rats did not induce a change in time spent "floating" (similar to immobility) in FST in stressed males and females tested in adolescence, but swimming and climbing behaviors were not measured (Toledo-Rodriguez and Sandi, 2011). The same 3-day stress paradigm as used in the present study (forced swim, elevated platform, and restraint) but applied during the pre-pubertal juvenile period (PND 27–29) resulted in no change in immobility in adult males and females, but similar to the present study, stress decreased climbing behavior in both males and females (Harris et al., 2022). This demonstrates that the timing, duration, and nature of stressors applied during adolescence impacts behavioral coping strategies in the FST (Holder and Blaustein, 2017).

Similar to previous reports, we found that males exhibited more climbing behavior compared to females in both adolescence and adulthood (Dalla et al., 2008; Drossopoulou et al., 2004; Harris et al., 2022; Hong et al., 2012). Another behavior with consistent sex differences in the FST is headshaking, the rapid side-to-side movement of head above the water, where males exhibit more headshaking behaviors than females (Kokras et al., 2017), which was true for both adolescents and adults in the present study. In male adolescents, but not adults, we found that pubertal stress decreased the number of headshakes during the FST compared to control males. Previous studies have shown that headshakes are positively correlated with serum testosterone levels and gonadectomy decreases head shaking to female levels (Kokras et al., 2017, 2018). Treatment with selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants can also decrease headshaking in male rats, suggesting that headshaking is also possibly regulated by serotonin and/or noradrenaline (Barros and Ferigolo, 1998; Kokras et al., 2015). The decrease in headshaking behavior in pubertal stress adolescent males suggests an antidepressant-like effect, however, this does not match the increased immobility and decreased climbing behavior also exhibited by pubertal stress adolescent males. The effects of these antidepressant drugs were studied in adult rats; therefore, it is unclear whether antidepressant treatment in adolescent males would cause similar decreases in headshaking.

Escape-oriented behaviors in the FST have been linked to certain monoamine neurotransmitter systems in studies of antidepressant drugs. Antidepressant drugs that increase the extracellular concentration of 5-HT, such as SSRIs, have been shown to increase swimming behavior but not climbing behavior in the rat modified forced swim test (Cryan et al., 2005a; Detke et al., 1995; Page et al., 1999). On the other hand, antidepressant drugs that increase the extracellular concentration of noradrenaline (e.g. desipramine) have been shown to increase climbing behavior but not swimming behavior (Cryan et al., 2002, 2005a; Kelliher et al., 2003). Further, depleting serotonin has been shown to prevent SSRI-induced increases in swimming behavior while having no effect on noradrenaline reuptake inhibitor-induced increases in climbing behavior (Page et al., 1999).

Within this context, one might expect that an increase in 5-HT or its turnover would result in increased swimming behavior, whereas a decrease would result in reduced swimming behavior. In agreement, we found that pubertal stress decreased swimming behavior specifically in adolescent females and, in a separate group of behavioral test-naïve rats, pubertal stress decreased 5-HT specifically in adolescent females, but not in adult females or in males of either age. However, it should also be noted that we found increased 5-HT turnover in adolescent females despite no significant effect on its metabolite 5-HIAA, which makes interpretation of the data more difficult. Pubertal stress led to an opposing effect in adolescent males, where serotonin turnover was *decreased* due to a decrease in the metabolite 5-HIAA without any effect on 5-HT, potentially indicating a sex-specific change in 5-HT metabolism in the adolescent brainstem in response to pubertal stress. The dorsal raphe nucleus (dRN), a principal source of 5-HT synthesis in the brain, is sensitive to stressful conditions due to the expression of corticotropin releasing factor (CRF) receptors (Lukkes et al., 2008). The opposing effects of pubertal stress on brainstem 5-HT turnover in adolescent males and females (decreasing turnover in males and increasing it in females) may stem from the sex difference in CRF receptors in the dRN, where female rats have greater expression of CRF_1 receptors compared to males in adolescence (Lukkes et al., 2016). CRF binding in the dRN increases activity of tryptophan hydroxylase, the rate-limiting enzyme for 5-HT synthesis (Hale and Lowry, 2011). Given the elevated expression of CRF receptors in the female dRN, this provides one possible mechanism by which pubertal stress increases brainstem 5-HT turnover in females.

In adult rats, we found that pubertal stress increased immobility in both sexes with no effect on swimming behavior. We therefore hypothesized that brainstem serotonin would be altered in a sex-independent manner. Indeed, the sex-specific effect of pubertal stress did not persist to adult brainstem 5-HT. Rather, there was an overall effect of pubertal stress whereby 5-HT turnover was decreased in both males and females, indicating an age-specific effect of pubertal stress on brainstem 5-HT. In our study, 5-HT turnover and metabolite concentrations from behavior-naïve rats cannot be directly correlated with FST behavior. However, the opposing direction of pubertal stress-induced changes in brainstem 5-HT turnover in females between adolescence and adulthood (increased in adolescence, but decreased in adulthood) points to a partial explanation of the shift in behavioral strategies used by pubertally stressed adolescent and adult females in the FST (decreased swimming in adolescence and no difference in swimming in adulthood). Future research should address potential pubertal stress-related alterations in the downstream targets of brainstem serotonergic innervation such as the mPFC, nucleus accumbens, and hippocampus. For example, would selective manipulation of 5-HT receptors replicate the sex-specific effect of pubertal stress on swimming behavior in adolescents?

As discussed above, antidepressant drugs that increase the extracellular concentration of noradrenaline have been shown to increase climbing behavior but not swimming behavior (Cryan et al., 2002, 2005a; Kelliher et al., 2003). Similarly, depleting noradrenaline prevents increased climbing behavior induced by antidepressants that act on noradrenaline while having no effect on SSRI-induced swimming behavior (Cryan et al., 2002). Given that pubertal stress decreased climbing behavior in adolescent males, but did not impact climbing behavior in adolescent females, we anticipated that pubertal stress would be associated with decreased brainstem noradrenaline concentrations in adolescent males. However, we found that pubertal stress counterintuitively decreased brainstem noradrenaline specifically in adolescent females compared to controls. Importantly, these rats had not undergone behavioral tests, and therefore the changes in brainstem noradrenaline cannot be directly linked to climbing behavior in the FST.

However, even in studies where endogenous brainstem noradrenaline is experimentally depleted, this manipulation alone does not impact climbing behavior in the FST (Cryan et al., 2002). This demonstrates that noradrenaline may modulate FST behavior only under specific circumstances, such as under antidepressant treatment.

In the transition from adolescence to adulthood, the locus coeruleus (LC, a brainstem noradrenergic nucleus that innervates the forebrain) increases its capacity to adapt to stressors and decreases spontaneous cell firing (Zitnik et al., 2016). Interestingly, the LC in females is larger and more stress sensitive in females compared to males (Bangasser et al., 2016; Lukkes et al., 2016) and undergoes sexual differentiation during puberty (Pinos et al., 2001). The sexually dimorphic nature of the LC fits with our finding that brainstem noradrenaline is higher overall in females than males in both adolescence and adulthood. The increased stress sensitivity of the female noradrenergic system also aligns with our finding that pubertal stress decreased brainstem noradrenaline concentrations specifically in females that were behavioral test-naive, despite a lack of a clear behavioral correlate.

4.5. Limitations

Because the goal of this study was to examine the impact of stress specifically during pubertal maturation, the stressors need to be applied at different chronological ages in males and females to align with the timing of puberty onset. However, this comes with the limitation that the sex-specific stress-induced behavioral changes may be due to a difference in chronological age, rather than pubertal maturation alone. Given that males underwent behavioral testing 10 days later than females, some of the sex differences observed in behavioral tests may be explained by this difference in chronological age. It is therefore unclear the extent to which the sex-specific nature of the behavioral effects of pubertal stress is a result of unique pubertal factors or the difference in chronological age at the time of stressor application and at behavioral testing. Further research is warranted to determine which critical windows of adolescent brain development are vulnerable to stress and furthermore, which of these stress-vulnerable critical windows are influenced by sex.

It should also be noted that the behavioral testing included periods of single housing, which can be stressful for rodents. Adolescents and adults were single housed for the 2 days of the SPT plus 4 days during the NIH test for adults only. However, since the control and pubertal stress groups of both sexes were all exposed to single housing, we cannot disentangle the potential interacting effects of social isolation and prior pubertal stress in the current study. To avoid a potential influence of behavioral testing, concentrations of monoamine neurotransmitters were quantified in brains from rats that did not undergo behavioral testing, so direct correlations with behavior (SPT, FST) cannot be made. Free-hand dissection of the brainstem and striatum is a somewhat crude method and limits the anatomical resolution. The collection of the whole striatum may have masked potential region-specific pubertal stress-induced alterations in dopamine in the dorsal versus ventral striatum, which would have differing behavioral impacts due to the distinct functions of the two subregions (Voorn et al., 2004). These limitations may explain why dopamine turnover alterations in striatum do not precisely align with saccharin preference behavior.

Quantification of neurotransmitters in tissue by HPLC can also limit functional implications because the method does not differentiate intracellular versus extracellular neurotransmitters/metabolites. Further, behavioral consequences of neurotransmitter concentration changes in one brain area may vary greatly depending on receptor availability and sensitivity. Nevertheless, these data do hint towards an important role of monoamine neurotransmitter systems in regulating the behavioral responses to pubertal stress. Future studies should further examine the specific mechanism by which pubertal stress impacts monoamine neurotransmitters and their receptors within discrete brain regions.

4.6. Conclusion

Together, these data suggest that stress applied specifically during the pubertal stage of adolescence exerts sex-specific effects on antidepressant-like behaviors, corticosterone, and monoamine neurotransmitters in adolescence and/or adulthood but it does not preferentially induce depressive-like behaviors in females compared to males. The persistence of pubertal stress-associated increases in immobility behavior in the FST, exploratory rearing in the OFT of adult females, and anhedonia in adult males suggest that a 3-day stress paradigm during puberty is sufficient to produce long-lasting neurobehavioral perturbations, which warrant further exploration. While this model did not produce the hypothesized pattern of female-biased vulnerability to depression that is observed in humans, it does provide additional evidence that responses to stress can be sex-specific. This further highlights the critical importance of examining both males and females in neuropsychiatric research to facilitate precision medicine.

CRediT authorship contribution statement

Erin P. Harris: Investigation, Formal analysis, Visualization, Methodology, Writing – original draft. **Francisca Villalobos-Manriquez:** Investigation, Formal analysis. **Thieza G. Melo:** Formal analysis. **Gerard Clarke:** Investigation, Formal analysis, Writing – review & editing. **Olivia F. O'Leary:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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