Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring

Min Hu[a,b,1], Jennifer Elise Richard[c,1], Manuel Maliqueo[a,d,1], Milana Kokosar[c], Romina Fornes[a], Anna Benrick[c], Thomas Jansson[e], Claes Ohlsson[f], Xiaoke Wu[b,2], Karolina Patrycja Skibicka[c,3], and Elisabet Stener-Victorin[a,2,3]

[a]Department of Physiology and Pharmacology, Karolinska Institutet, 17177 Stockholm, Sweden; [b]Department of Obstetrics and Gynecology, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, 150040 Harbin, China; [c]Department of Physiology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, 405 30 Gothenburg, Sweden; [d]Endocrinology and Metabolism Laboratory, Department of Medicine West Division, School of Medicine, University of Chile, 8320000 Santiago, Chile; [e]Department of Obstetrics & Gynecology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045; and [f]Centre for Bone and Arthritis Research, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, 413 45 Gothenburg, Sweden

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During pregnancy, women with polycystic ovary syndrome (PCOS) display high circulating androgen levels that may affect the fetus and increase the risk of mood disorders in offspring. This study investigated whether maternal androgen excess causes anxiety-like behavior in offspring mimicking anxiety disorders in PCOS. The PCOS phenotype was induced in rats following prenatal androgen (PNA) exposure. PNA offspring displayed anxiety-like behavior in the elevated plus maze, which was reversed by flutamide [androgen receptor (AR) blocker] and tamoxifen [selective estrogen receptor (ER) modulator]. Circulating sex steroids did not differ between groups at adult age. The expression of serotonergic and GABAergic genes associated with emotional regulation in the amygdala was consistent with anxiety-like behavior in female, and partly in male PNA offspring. Furthermore, AR expression in amygdala was reduced in female PNA offspring and also in females exposed to testosterone in adult age. To determine whether AR activation in amygdala affects anxiety-like behavior, female rats were given testosterone microinjections into amygdala, which resulted in anxiety-like behavior. Together, these data describe the anxiety-like behavior in PNA offspring and adult females with androgen excess, an impact that seems to occur during fetal life, and is mediated via AR in amygdala, together with changes in ERα, serotonergic, and GABAergic genes in amygdala and hippocampus. The anxiety-like behavior following testosterone microinjections into amygdala demonstrates a key role for AR activation in this brain area. These results suggest that maternal androgen excess may underpin the risk of developing anxiety disorders in daughters and sons of PCOS mothers.

maternal androgen excess | anxiety | behavior | polycystic ovary syndrome | amygdala

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder characterized by excessive androgen secretion and abnormal insulin activity and affects up to 17% of women worldwide (1). Women with PCOS are at an increased risk of developing symptoms of anxiety and depression. In fact, over 60% of women with PCOS are diagnosed with at least one psychiatric disorder, such as depression, anxiety, or an eating disorder (2). Suicide attempts have also been shown to be seven times more common in women with PCOS than in healthy controls (3). The mechanisms underlying the development of PCOS are poorly understood. Although a genetic basis for PCOS has been suggested, the intrauterine milieu might also affect the reproductive/endocrine function of a child born to a PCOS mother in a manner that is independent of genetic inheritance or sex. It is also known that daughters of mothers with PCOS are at increased risk of developing the syndrome and that sons tend to suffer from obesity and insulin resistance (4). Thus, it has been proposed that PCOS originates during fetal development and that this might be, in part, a result of maternal androgen excess (5).

Maternal testosterone levels in humans have been shown to affect brain morphology and function (6) and to be correlated to neural development and mental function (7). There is evidence for a crucial role of the hippocampus and the amygdala in the development of anxiety and depression, and that these neural circuits are affected by fluctuations in sex steroids in humans and in rodents (8). We have previously demonstrated that continuous exposure to dihydrotestosterone (DHT) from puberty until adulthood in female rats down-regulates androgen receptor (AR) expression in the hypothalamus and induces anxiety-like behavior in female rats (9). The increased rates of anxiety disorders and disruptive behavioral disorders among children with genetically induced hyperandrogenism further indicate that androgen excess may contribute to a higher risk of psychopathology (10).

During pregnancy, androgens are metabolized to estrogens by the placenta in women and by the ovaries in rodents. Thus, the effects of testosterone on pregnancy are partly mediated by estrogen (11). Women with PCOS exhibit high circulating androgen levels during pregnancy, which hypothetically could be related to the increased risk of mood disorders in their offspring (12).

Significance

Polycystic ovary syndrome (PCOS) is the leading cause of anovulatory infertility characterized by excessive androgen secretion. PCOS women are at an increased risk of developing depression and anxiety disorders. Although the etiology of PCOS is unclear, it is proposed to originate during fetal development because of maternal androgen excess. We describe here, in rodent models reflecting the anxiety phenotype of PCOS, evidence for disordered androgen receptor function in the amygdala, together with changes in estrogen receptor-α, serotonergic and GABAergic genes in the amygdala, and hippocampus. These findings define a previously unknown mechanism that may be critical in understanding how maternal androgen excess has the potential to increase the risk of developing anxiety disorders in daughters and sons of PCOS mothers.

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[1]M.H., J.E.R., and M.M. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: xiaokewu2002@vip.sina.com or Elisabet.stener-victorin@ki.se.

[3]K.P.S., and E.S.-V. contributed equally to this work.

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Here, we tested the hypothesis that an excess of androgens in dams during pregnancy may cause anxiety-like behavior in adult female and male offspring. We used the prenatal androgen (PNA) model, which mimics the elevation of androgens in women with PCOS during pregnancy (13). The phenotype of the PNA model in mice (14) and in rats (12) reflects reproductive and metabolic characteristics of lean women with PCOS. However, whether it reflects symptoms of anxiety (2) is unknown. We demonstrated that female PNA offspring exhibited increased anxiety-like behavior, which was prevented by blocking the AR during pregnancy, implicating AR-mediated signaling in mediating the altered behavior of PNA offspring. To understand the neuroanatomical distribution of sites affected by the PNA treatment we evaluated the gene expression of key steroid receptors [*Ar*, estrogen receptor-α (*Erα*), *Erβ*, and G protein-coupled estrogen receptor (*Gper*)] in the hypothalamus, hippocampus, and amygdala, brain areas known to be involved in the regulation of mood behavior in female offspring. The expression of the AR gene was selectively altered in the amygdala of the PNA offspring. We further show that subchronic testosterone exposure in adult females also reduced *Ar* expression in the amygdala. Because the amygdala is known to be involved in the regulation of mood behavior, we hypothesized that testosterone exerts an anxiogenic effect in the amygdala. We obtained support for this hypothesis by demonstrating that intra-amygdala testosterone microinjections resulted in anxiety-like behavior.

Materials and Methods

Detailed materials and methods are provided in *SI Materials and Methods*.

Experiments 1 and 2.

Animals. Pregnant Wistar rats (Charles River) arrived on gestational day (GD) 8 and were housed in individual cages on a 12-h light/dark cycle, with a temperature of 21–22 °C at 55–65% humidity. They received ad libitum access to water and standard chow (no. 2016; Harlan Winkelmann). All studies were carried out with ethical permissions from the Animal Ethics Committee of the University of Gothenburg (Ethical no: 53-2013 and 195-2013), in accordance with legal requirements of the European Community (Decree 86/609/EEC).

PNA treatment. Pregnant dams were randomly assigned to one of four groups and treated with daily subcutaneous injections [from GD 15–19 (n = 5 per group)] of: (*i*) vehicle; (*ii*) testosterone (T): T 0.5 mg·kg·d; (*iii*) testosterone + flutamide (T + Flut): T 0.5 mg·kg·d with flutamide 7.5 mg·kg·d; and (*iv*) testosterone + tamoxifen (T + Tam): T 0.5 mg·kg·d with tamoxifen 10 μg·kg·d.

Experiment 1. On GD21, dams were anesthetized and maternal and placental samples were collected. Blood from dams was centrifuged, and the serum was frozen at –80 °C for subsequent measurement of sex steroids.

Experiment 2.

Phenotyping of female offspring. The estrous cycle phase was determined by vaginal smears. Ovaries were dissected and fixed for ovarian morphology. Euglycemic hyperinsulinemic clamp was performed to evaluate insulin sensitivity.

Postnatal weight development and food intake in offspring. Pups were weighed from day 4 until adult age. Sex was confirmed by genotyping and female and male offspring were separated from dams at day 21. Body weight and food intake were measured weekly until behavioral testing or clamp.

Behavioral testing. For details see *SI Materials and Methods*. To investigate the presence of anxiety-like behavior in male and female offspring of PCOS dams the elevated plus maze (EPM) test was performed. Locomotor activity was tested immediately after the EPM for 30 min in photo-cell equipped activity boxes (Kungsbacka Mätoch Reglerteknik).

Tissue collection and sex-steroid and corticosterone analyses. After the last behavioral test and the euglycemic-hyperinsulinmic clamp, rats were killed by decapitation and tissues dissected and snap-frozen.

RNA isolation and mRNA expression. Tissues from the hypothalamus, hippocampus and amygdala were analyzed in female and male offspring of PCOS dams. TaqMan probe sets for target genes and reference genes were chosen from the online catalog (Table S1). Gene expression was quantified relative to the housekeeping genes *β-actin* and *Gapdh*. Genes examined were selected because of their role in the regulation of steroidal hormones that may affect the development of PCOS as well as genes with a role in anxiety-like behavior in these brain areas. Table S2 shows mean of target gene C_T value.

Experiment 3.

Animals and brain cannula surgery. Adult female Wistar rats (7–8 wk of age) (Charles River) were housed in individual cages in a 12-h light/dark cycle with free access to chow and water. Brain cannula surgery was performed as described in ref. 15.

Behavioral testing and intra-amygdala microinjections. Details of intra-amygdala microinjection of testosterone including dosing and behavioral testing is described in *SI Materials and Methods*.

Experiment 4. Adult female Wistar rats (8–9 wk of age) were treated with daily subcutaneous injection of: (*i*) vehicle (n = 12); (*ii*) T 0.5 mg·kg·d (n = 14). After 4 d animals were tested in the diestrus phase for 5 min on the EPM and again 8 d of treatment on the EPM and in the open field, followed by dissection of amygdala and mRNA expression analyses.

Statistical Analysis. All data are presented as mean ± SEM. The Kruskal–Wallis test followed by Mann–Whitney U test or one-way ANOVA followed by Dunnett's post hoc test was used in Exp. 1 and 2. Two-way ANOVA was used to analyze behavior in Exp. 2. The independent-samples t test was used for group comparisons of behavioral activity and relative gene expression, one-way ANOVA repeated measurement was used for body weight change and food intake in Exp. 3 and 4 (SPSS version 21.0; SPSS, and Prism GraphPad version 6.0, GraphPad Software). P values lower than 0.05 were considered statistically significant.

Results

Experiment 1. Testosterone-treated pregnant dams had elevated circulating testosterone and DHT and decreased placental weight (Table S3). Placentas from testosterone-treated dams displayed the same pattern as in women with PCOS characterized by increased phosphorylation and total STAT3 protein expression compared with vehicle-treated dams (Fig. S1). Tamoxifen further increased pSTAT3 and STAT3. Thus, the animal model used here displays a lean PCOS-like feature from a maternal and placental perspective.

Experiment 2.

Phenotyping of female offspring. The phenotypic presentation of female offspring of T-treated dams is presented in *SI Materials and Methods*, with Fig. S2 and Table S4 demonstrating that the model, in part, mimics human PCOS, and reproduces the irregular cycles and PCO morphology, albeit not elevated circulating testosterone.

Postnatal body weight and food intake. Body weight development and food intake of female and male offspring of T-treated dams is presented in Fig. S3.

Anxiety-like behavior. Female offspring of T-treated dams spent significantly less time in the open arm ($P < 0.05$) and more time in the closed arm ($P < 0.01$) in the EPM compared with vehicle-treated animals (Fig. 1 *A* and *B*). This behavior was reversed by AR blockade and selective ER modulator (SERM) because time spent in open and closed arms in T+Flut and T+Tam-treated rats did not differ from vehicle-treated female rats (Fig. 1 *A* and *B*). These effects were observed without changes in total horizontal locomotor activity (Fig. 1*C*). One-way ANOVA indicated no significant changes in behavior in male PNA rats (Fig. 1 *D*–*F*). However, as shown in detail in *SI Materials and Methods*, when data from male and female offspring are analyzed together by two-way ANOVA (sex and treatment), there was a main effect of treatment on both time in the open and closed arm and this effect was reversed by the AR blockade and the SERM. Thus, the PNA effect on anxiety may not be restricted to female offspring.

Gene expression analysis. Because of anxiety-like behavior exhibited by female and possibly male PNA offspring, we analyzed gene expression in the hypothalamus, hippocampus, and amygdala, three important areas in the regulation of anxiety-like behavior, as gene expression of AR and ER in these brain nuclei may play an important role in emotionality and energy balance regulation. No significant differences were found in the mRNA expression of *Ar*, *Esr1* (ERα), *Esr2* (ERβ), and *Gper1* in the hypothalamus between PNA and vehicle-treated female offspring (Fig. 2*A*). The hypothalamic mRNA expression of *Ar* was slightly lower in

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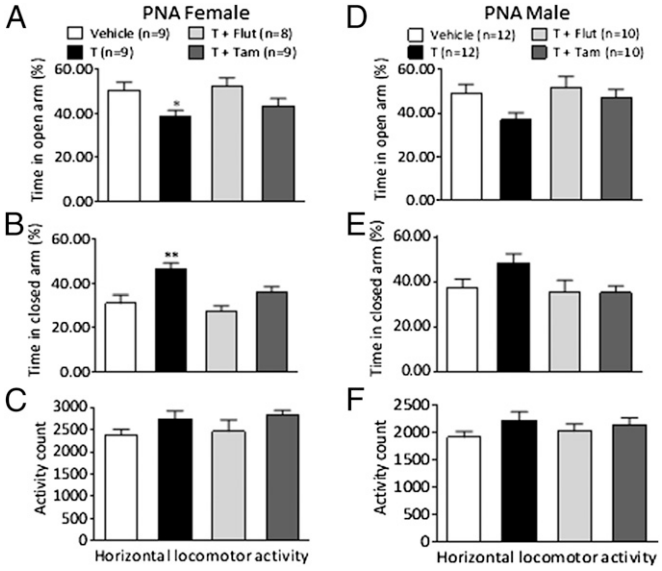

Fig. 1. Anxiety-like behavior in female and male offspring of T-treated dams (Exp. 2). Time (%) spent in (*A* and *D*) open arms and (*B* and *E*) closed arms in the EPM in female offspring (*A* and *B*) and male offspring (*D* and *E*). Horizontal locomotor activity in females (*C*) and males (*F*). Values expressed as means ± SEM; $*P < 0.05$, $**P < 0.01$ compared with vehicle-treated group.

male PNA offspring than in vehicle-treated (Fig. 2*D*). There was a 50% reduction of *Esr1* mRNA expression in the hippocampus ($P < 0.05$); in contrast, the expression of *Gper1*, *Ar*, and *Esr2* were not different in female PNA offspring compared with control animals (Fig. 2*B*). *Ar* mRNA expression in the amygdala was decreased by ∼50% in female PNA rats compared with vehicle-treated offspring ($P < 0.01$), but there were no significant differences in the expression of *Esr1*, *Esr2*, or *Gper1* compared with vehicle-treated rats (Fig. 2*C*). There were no differences in the gene expression of AR and ERs in the hippocampus and amygdala of male PNA offspring (Fig. 2 *E* and *F*).

As the initial analysis revealed significant differences in steroid-related gene expression in the amygdala and the hippocampus, we next investigated whether serotonergic and GABAergic receptors and enzymes involved in anxiety-like behavior were affected in these areas in PNA offspring. The mRNA expression of 5-hydroxytryptamine (serotonin) receptor 2C (*Htr2c*) was more than twofold increased in the amygdala of PNA female rats compared with control rats ($P < 0.001$), whereas *Htr1a* did not differ (Fig. 3*C*). There were no changes in serotonin receptor gene expression in male PNA offspring (Fig. 3*G*). There was a marked reduction in the expression of *Htr1a* in the hippocampus ($P < 0.05$), with no difference in *Htr2c* expression in female PNA offspring in this brain area (Fig. 3*A*). There was a pronounced increase in *Htr2c* expression in the hippocampus of PNA male rats compared with controls (Fig. 3*E*). Next, we analyzed the expression of genes related to GABAergic signaling and synthesis as they were previously shown to be crucial in the modulation of anxiety behavior. The amygdala and hippocampus mRNA expression of glutamate decarboxylase genes, *Gad1* and *Gad2*, enzymes necessary for the production of the inhibitory neurotransmitter GABA, was not affected by PNA treatment in female (Fig. 3 *B* and *D*) or male offspring (Fig. 3 *F* and *H*). $GABA_B$ receptors have been reported to be involved in the modulation of anxiety-like behavior (16). The *Gabbr1* expression was increased threefold in the amygdala of female PNA rats ($P < 0.001$) (Fig. 3*D*); in male PNA rats, a much less-pronounced decrease was detected ($P < 0.05$) (Fig. 3*H*).

In addition, we compared hypothalamus, hippocampus, and amygdala gene expression of vehicle-treated male and female offspring with testosterone-treated female offspring. With this analysis we demonstrate that the expression of a serotonergic receptor, *Htr1a*, found at higher levels in control female offspring compared with control male offspring, is reduced in females by maternal testosterone treatment, indicating that the maternal testosterone dose used may masculinize the brain in female offspring (Fig. S4 *D* and *E*).

Experiment 3. In light of the more pronounced anxiety-like behavior exhibited by female PNA offspring and the critical role that the amygdala plays in the regulation of anxiety combined with the profound changes in *Ar*, *Htr2c*, and *Gabbr1* expression in this area induced by PNA treatment in females but not males, we set out to determine whether AR activation, specifically in the amygdala, induces anxiety-like behavior in females. Intra-amygdala microinjections of testosterone were centered at the basolateral part of the amygdala (Fig. 4*A*). There were no changes in anxiety-like behavior 1 and 24 h after intra-amygdala microinjections of 10 μg testosterone (Fig. 4 *D*–*G*). Anxiogenic effects of the intra-amygdala testosterone treatment (10 μg daily) emerged after 4–5 d of treatment, and persisted throughout the second estrus cycle (8–10 treatments), where the dose was increased to 20 μg. Intra-amygdala testosterone-treated female rats spent significantly less time in the open arm and longer time in the closed arm of the EPM compared with vehicle-treated rats (Fig. 4*J*). In the open-field test, testosterone-treated rats spent significantly less time in the central area and more time in the periphery than control females (Fig. 4 *I* and *K*), without differences in locomotor activity (Fig. S5 *A*–*D*). There was no difference in body weight gain or food intake between control and testosterone-treated females receiving daily microinjections into the amygdala (Fig. 4 *B* and *C*). The intra-amygdala testosterone injection in adult female rats did not significantly affect the cycle length.

Experiment 4. To link the anxiety-like behavior in female PNA offspring and in adult females exposed for intra-amygdala testosterone, we further investigated whether adult testosterone exposure alters amygdala AR expression. Adult female rats were subcutaneously injected with testosterone for 8 d. Testosterone-treated female rats spent significantly less time in the open arm ($P < 0.01$), and longer time in the closed arm ($P < 0.05$), with no differences in locomotor activity (Fig. 5*A* and Fig. S6 *A*–*C*). The *Ar* mRNA expression in the amygdala was decreased by ∼30% in testosterone-treated adult females compared with controls ($P < 0.01$), with no differences in *Esr1, Esr2*, or *Gper1* expression (Fig. 5*D*). Testosterone-treated females had a disrupted estrus cycle after 4–5 d of testosterone exposure and gained more weight than controls during the treatment (Fig. S6*D*).

Discussion

These findings reveal a brain mechanism potentially underpinning anxiety disorder in women with PCOS. We find that maternal

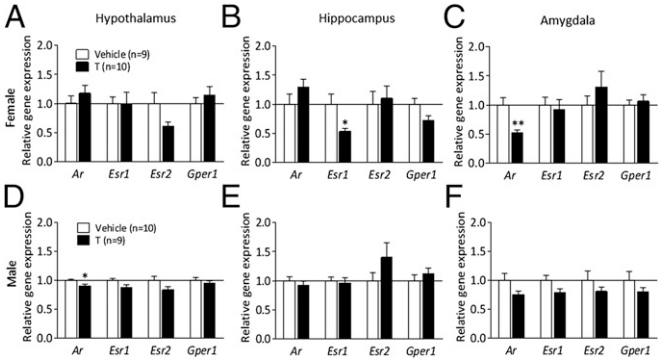

Fig. 2. Gene expression of androgen and estrogen receptors (Exp. 2) in (*A*) hypothalamus, (*B*) hippocampus, and (*C*) amygdala in female PNA offspring, and in (*D*) hypothalamus, (*E*) hippocampus, and (*F*) amygdala in male PNA offspring. Values represent means ± SEM; $*P < 0.05$, $**P < 0.01$ vs. the vehicle-treated group.

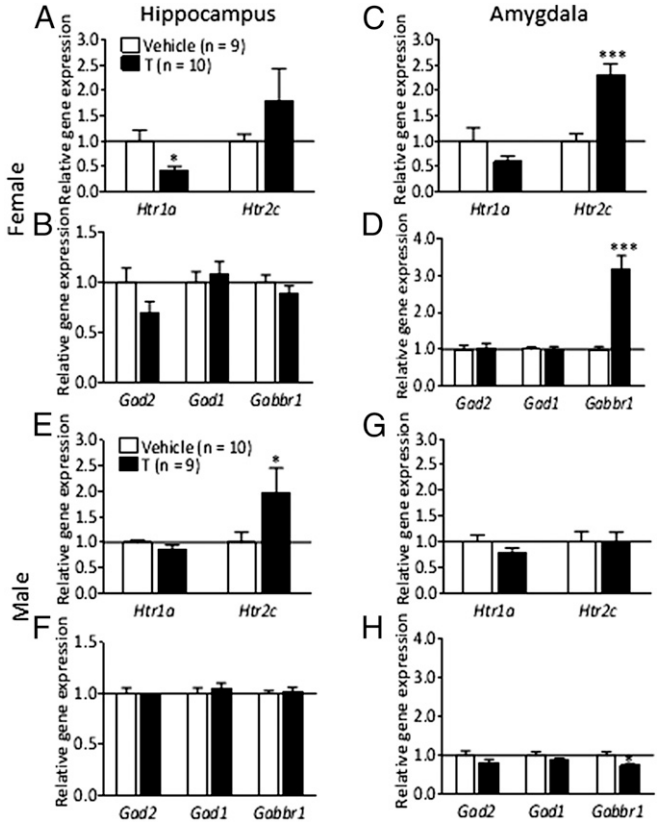

Fig. 3. Expression of serotonergic and GABAergic genes (Exp. 2) in the hippocampus (*A* and *B*) and amygdala (*C* and *D*) of female offspring, and in the hippocampus (*E* and *F*) and the amygdala (*G* and *H*) of male offspring. Values are means ± SEM; $*P < 0.05$, $***P < 0.001$ vs. vehicle-treated group.

androgen excess results in anxiety-like behavior in female PNA offspring, which is associated with decreased gene expression of *Ar* and increased expression of GABAergic and serotoninergic receptors in the amygdala, consistent with an increased anxiety-like behavior. We further show that subchronic exposure of adult females to testosterone also reduces amygdala *Ar* expression. Activation of AR restricted to the amygdala, by intra-amygdala microinjections of testosterone, was sufficient to induce anxiety-like behavior in females, demonstrating a new role for amygdala AR. These experiments suggest that the anxiety-like behavior in female PNA offspring may be mediated by direct effects of testosterone on fetal amygdala. Although no changes in behavior in male PNA rats were observed, when taking sex and treatment into consideration, both female and male PNA offspring display anxiety-like behavior.

Women with PCOS have an increased prevalence of anxiety and depression symptoms (2, 17), which is independent of body mass index. Clinical studies have previously shown that the levels of circulating free testosterone are lower in women with symptoms of depression, but no associations with symptoms of anxiety were observed (18). Although low, rather than high, circulating testosterone levels are associated with more symptoms of depression, testosterone was higher in these individuals than in women without PCOS (18, 19). Importantly, other investigators have reported higher testosterone concentrations in women with severe clinical depression (20), suggesting that testosterone may cause mood disturbances. Furthermore, there seems to be a link between estrogens and symptoms of depression, although no differences were found between serum estradiol levels in women with and without depression (21). Although clinical data are inconsistent, there are indications that androgens play a crucial role in behavior and mood regulation in females. Of note, women with PCOS are not only hyperandrogenic, many are also hyperestrogenic (19). By using testosterone we wanted to closely mimic the elevated testosterone levels in women with PCOS that could potentially result in activation of both androgenic and estrogenic pathways. Although our approach cannot separate the androgenic effect alone, it better represents the pathophysiology of PCOS.

Women with PCOS, and their daughters, have an increased risk of metabolic dysfunction, impaired quality of life, and infertility. Whether these women suffer from psychiatric-ill health is not well established. In an experimental setting, where autonomic activity and mood were assessed in healthy women exposed to testosterone or placebo, women exposed to testosterone exhibited an elevated anxiety-prone response (22). Chronic exposure to testosterone also leads to increased anxiety in female mice, an effect associated with increased levels of corticotropin-releasing factor (23). In the present study, female offspring of dams treated with testosterone during pregnancy, as well as adult females treated with intra-amygdala testosterone, displayed increased anxiety-like behavior. Importantly, the anxiety-like behavior in female PNA offspring was independent of changes in body weight, food intake, and circulating corticosterone levels.

Studies on the link between testosterone and anxiety behavior in males have generated inconsistent results (24, 25). In our study, male PNA offspring displayed anxiety-like behavior pattern similar—although less prominent—to female offspring, mirroring previous human and animal studies (26).

Several possibilities exist for a potential mechanism by which high circulating testosterone in the mother ultimately changes emotionality behavior in the offspring. One possible explanation may be an effect of maternal testosterone exposure on the organization of the brain, which may permanently alter brain morphology and subsequently give rise to altered behavioral responses (27). Another possibility is that high testosterone in the mother alters steroid production in the placenta (12), which may affect the fetus. We found that placental weight was reduced in androgenized rats. The increase in placental total and phosphorylated STAT3 in pregnant rats exposed to testosterone or testosterone + SERM indicate an androgenic effect, which is not mediated by estrogen receptors. The consequences of increased placental STAT3 phosphorylation remain to be established, but may include activation of key placental amino acid transporters (28). It is interesting to note that STAT3 activation is increased in placenta from women with PCOS and in obese women (13). In both conditions the offspring develops insulin resistance and metabolic disturbances in the adult life (4), as in the present study.

If maternal androgen excess with altered placenta function results in offspring with altered testosterone levels, it could be the main culprit behind the increased anxiety. To our knowledge, our study is the first to use the highly sensitive and specific gas chromatography (GC)-MS/MS to analyze circulating sex steroids in female rat PNA offspring. We found no differences between the experimental groups. These results are in line with a recent study in the offspring of female PNA mice that used liquid chromatography tandem (LC)-MS/MS (29). In contrast, higher circulating testosterone has previously been reported in female rat PNA offspring (12). This discrepancy may be a result of the higher doses of maternal testosterone (5 mg) used in the previous study compared with the present study (0.5 mg). The association between circulating testosterone and mood disorders has been suggested to be U-shaped, in which too-high or too-low levels cause behavioral dysfunction (30). Although the anxiety-like behavior observed in the female PNA offspring in the present study cannot be directly explained by high circulating androgens, the reduced AR expression in the amygdala suggests a compensatory response to the high prenatal testosterone exposure, a result implicating the amygdala as the CNS site underlying the changes in anxiety in the PNA offspring. This idea is further strengthened by our experiment showing that subchronic testosterone exposure into amygdala is sufficient to produce anxiety-like behavior in adult females. Furthermore, we also show that a compensatory reduction

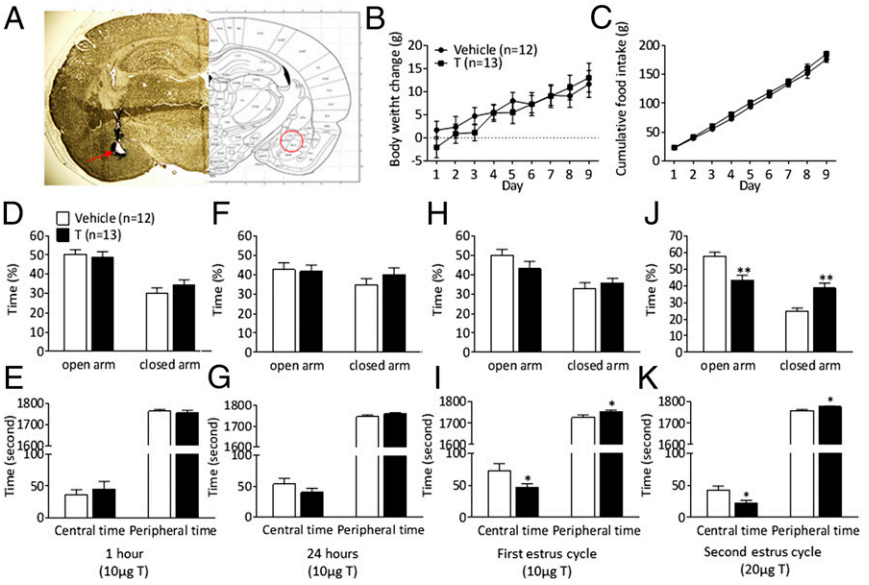

Fig. 4. Intra-amygdala testosterone application produces anxiety-like behavior in adult females (Exp. 3). EPM and open-field test were performed after subchronic daily testosterone microinjections into the amygdala during two estrus cycles. (*A*) Histological verification of the location of the cannula in the amygdala. (*Right*) Photomicrograph of a 40-μm coronal section of a rat brain at the level of bregma −2.80 mm (i.e., microinjection site). (*Left*) Schematic representation of the amygdala. Body weight change (*B*) and cumulative daily food intake (*C*) during microinjections of testosterone or vehicle into the amygdala. EPM and open-field test 1 h (*D* and *E*) and 24 h (*F* and *G*) after vehicle ($n = 12$) or testosterone ($n = 13$) microinjections. The EPM and open-field test during the first (*H* and *I*) and second diestrus phase (*J* and *K*). Time spent in the open and closed arms during the EPM test (*D*, *F*, *H*, *J*). Time spent in central and peripheral area in the open field test (*E*, *G*, *I*, *K*). Values are expressed as means ± SEM; $*P < 0.05$, $**P < 0.01$ vs. the vehicle-treated group.

of amygdala *Ar* expression can also be driven by exposure of adult females to subchronic peripheral testosterone injections.

Increased anxiety-like behavior in PNA-females was prevented by flutamide or tamoxifen administration, indicating that both AR and ER receptors are involved in mediating the effect of PNA on anxiety. Whether these changes are in part mediated via aromatization of testosterone and therefore an estrogenic action remains to be established. Our findings demonstrate that anxiety-related behavior in female PNA offspring is associated with a decrease in mRNA expression of *Esr1* in the hippocampus. This finding is consistent with previous observations where ER antagonists injected into the hippocampus, but not amygdala, increase anxiety-like behavior in female rats (31).

Previous findings have indicated that testicular feminization, through mutations of the AR in male rats, causes anxiety-like behavior, further supporting the involvement of AR in anxiety-like behavior (32). In the present study, *Ar* expression was decreased in the hypothalamus of male PNA rats, with no changes in the expression of any steroid receptors measured in the amygdala.

In the present study, hypothalamic *Ar* expression was not altered in female PNA offspring, suggesting AR outside the hypothalamus may underlie emotionality responses. AR are also ubiquitously expressed in the limbic system, including in the amygdala (32). *Ar* expression was altered only in amygdala of the female PNA rats, indicating that AR in this area are uniquely sensitive to the increased exposure of maternal androgens. These results are in stark contrast to those obtained in males and indicate a differential response to prenatal androgen exposure in male versus female offspring. To understand testosterone's role in the regulation of anxiety-like behavior, testosterone was microinjected into this brain area and anxiety-like behavior was measured. Females given daily microinjections of testosterone into the amygdala displayed increased anxiety-like behavior in both the EPM and the open-field test. The onset of the anxiogenic effect required several days of treatment, indicating that synthesis of new proteins or even synaptic reorganization may be required for the anxiogenic effect. Of note, peripheral testosterone injections in adult females resulted in anxiety-like behavior already after 4 d of testosterone administration; increased anxiety persisted through the 8 d of treatment. Even in adult females, 8 d of testosterone exposure was sufficient to reduce *Ar* expression in the amygdala. Thus, both in utero and adult elevation of testosterone can impact on the amygdala and increase anxiety-like behavior.

Central serotonin and GABA signaling are well established for their role in the regulation of anxiety-like behavior, and both may be affected in PNA female offspring. *Htr1a* and *Htr2c* are two receptors that are included in a subfamily of serotonin receptors that are associated with mood regulation. Pharmacological blockade of *Htr1a* receptors can be anxiolytic because antagonists to these receptors have been shown to relieve anxiety and depression (33). *Htr2c* receptors are distributed throughout the central nervous system and have been linked to several behavioral responses, including depression, anxiety, and feeding behavior (34). In this study we found an increase of ∼50% of *Htr2c* expression in the amygdala and a reduction of *Htr1a* expression in the hippocampus. These observations are in line with previous studies demonstrating that activation of the *Htr2c* and depletion of *Htr1a* results in anxiety-like behavior in mice (35). Thus, the increase in *Htr2c* receptor expression in the amygdala of female PNA offspring is highly

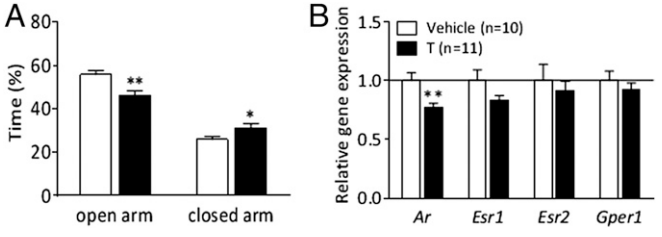

Fig. 5. Anxiety-like behavior and amygdala steroid receptor expression in adult female rats treated with 0.5 mg·kg·d testosterone subcutaneously during 8 d (Exp. 4). Time (%) spent in open arms and closed arms in the EPM at (*A*) second diestrus phase. Gene expression of androgen and estrogen receptors (*B*). Values expressed as means ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with vehicle-treated group. *Ar*, androgen receptor; *Esr1*, ERα; *Esr2*, ERβ; *Gper1*, G protein-coupled estrogen receptor 1; T, testosterone.

consistent with an increased anxiety profile. Of note, this was not replicated in males. In fact, in males *Htr2c* receptor expression was reduced in the hippocampus, a change more in line with an improved emotionality profile (36). That the maternal testosterone dose used can have a masculinizing effect on the female brain is supported by the finding that the amygdala and hippocampus expression of *Htr1a* is similar in control males and T-treated female offspring, but higher in control females.

Supraphysiological doses of androgens cause anxiety-like behavior in female mice and enhance presynaptic release of GABA (37). Anxiety-related behavior has also been associated with altered GABAergic transmission in the amygdala, including altered expression of GABA-synthesizing enzymes GAD65 (*Gad2*), GAD67 (*Gad1*), and GABA receptors. In the present study, amygdala *Gabbr1* mRNA expression was elevated in PNA female offspring. Previous studies demonstrated that $GABA_B$ receptor activation might provide a useful strategy in the treatment of anxiety-like behavior (16), thus we can only speculate that the change detected here may be in response to lower activity at this receptor in female PNA rats. Alterations in GABAergic receptors may also be expected because testosterone metabolites act as GABA receptor agonists (38). Taken together, these findings indicate that maternal androgen excess may cause anxiety-like behavior in daughters and sons of PCOS mothers, and that it may be because of altered amygdala AR, GABAergic, and serotonergic signaling.

In conclusion, maternal testosterone exposure causes anxiety-like behavior in female, and to a lesser extent male offspring, an effect that seems to occur during fetal life and to be mediated via AR in the amygdala, together with changes in ERα and in the serotonergic and GABAergic pathways in the amygdala and hippocampus of female PNA rats. To our knowledge, this is also the first study to show that testosterone has an anxiogenic role in females at the level of the amygdala. Collectively, our results suggest that maternal androgen excess may contribute to a greater risk of developing anxiety disorders in women with PCOS.

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