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Effects of androgen and leptin on behavioral and cellular responses in female rats ☆

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ABSTRACT

The causes of anxiety and depression in women with polycystic ovary syndrome (PCOS) remain elusive. To identify steps linking androgen signaling to the regulation of affective symptoms in vivo, we compared behavioral responses in female rats continuously exposed to DHT from puberty (a model of DHT-induced PCOS) and in rats exposed to DHT for 1 week.

Continuous and 1 week of DHT exposure resulted in a general decrease in locomotor activity and time spent on the open arms in the elevated plus maze, indicating anxiety-like behavior. Rats with DHT-induced PCOS have increases in adiposity and circulating leptin levels accompanied by leptin resistance. One week of DHT exposure decreased androgen receptor (AR) expression in the hypothalamus and leptin synthesis and function in adipocytes; it also inhibited signal transducer and activator of transcription 3 (STAT3) and attenuated leptin activity by increasing levels of soluble leptin receptor, a leptin-binding protein, in the hypothalamus. This may affect the androgen-induced anxiety-related behavior in female rats.

In conclusion, our results highlight the central role of androgens in behavioral function in female rats and suggest that androgens directly regulate the AR by decreasing its hypothalamic expression. Androgens also increase leptin synthesis in adipocytes, which drives central leptin signaling, and may regulate anxiety-related behaviors. Elucidating mechanisms by which androgens modulate female anxiety-like behavior may uncover useful approaches for treating women with PCOS who have symptoms of anxiety.

Introduction

Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism with elevated circulating androgens or clinical manifestations of androgen excess (Norman et al., 2007). Overweight/obesity, insulin resistance, and dyslipidemia may contribute to hyperandrogenism (Yildiz et al., 2008). Women with PCOS have compromised psychological health, but the pathophysiological correlates are unclear. It is not known whether symptoms of anxiety and depression in women with PCOS (Jedel et al., 2010a) are associated with the biochemical hyperandrogenism (Adali et al., 2008; Hollinrake et al., 2007; Mansson et al., 2008; Rasgon et al., 2003; Weiner et al., 2004). Symptoms of depression may be influenced by the balance of estrogen/testosterone concentrations, which fluctuate with age in women (Rohr, 2002). How activation of androgen signaling translates into anxiety/depression at the cellular and behavioral levels in females is not fully understood.

Androgens have diverse roles in reproduction and affect brain development and behavior (Foecking et al., 2008). For example, treatment with testosterone decreases anxiety and aggression in young, gonadectomized male mice (Frye et al., 2008). Through their interactions with androgen receptors (ARs), androgens orchestrate complex biological effects (Gao et al., 2005). Disruption of the AR gene results in the absence of male-typical aggressive and sexual behavior in mice (Walters et al., 2009). Male mice lacking the brain-specific AR do not show normal male-typical behavior or aggressive behaviors during dihydrotestosterone (DHT) exposure (Raskin et al., 2009). Thus, androgens may activate brain ARs to regulate behaviors in males.

In women, androgen therapy improves depressive states (Rohr, 2002) and spatial memory (Postma et al., 2000). Although central androgen/AR signaling contributes to behavioral changes in a positive

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way, women with PCOS and androgen excess display symptoms of anxiety (Benson et al., 2009; Deeks et al., 2010; Jedel et al., 2010a) and depression (Hollinrake et al., 2007; Kerchner et al., 2009; Rasgon et al., 2003). Thus, it is unclear whether high circulating androgens are associated with depression and anxiety symptoms in women with PCOS (Adali et al., 2008; Weiner et al., 2004). Recently we demonstrated that lower circulating free testosterone and a glucuronidated androgen metabolite, rather than higher, are associated with worse self-reported depression symptoms in women with PCOS (Jedel et al., 2011). Whether there is an association between androgens and symptoms of anxiety and depression requires further exploration in PCOS.

Adipocyte-derived leptin, encoded by the obese gene (*ob*) (Myers et al., 2008), is a crucial peripheral factor acting on central networks that modulate food intake, cognition, learning, and other behaviors (Leshan et al., 2006). Leptin has an antidepressant-like effect in male rats (Lu et al., 2006), but its pathophysiologic role in psychiatric disorders, particularly depression, in humans is unclear (Taylor and Macqueen, 2010). Women with PCOS have elevated circulating leptin levels, especially the obese women (Pusalkar et al., 2010), but it is not known whether leptin is associated with anxiety and depression in PCOS. However, it is known that women with comorbid PCOS and depression are more obese and insulin resistant compared to women with PCOS without depression (Hollinrake et al., 2007).

Previously, we have phenotyped female rats exposed to the nonaromatizable androgen 5α-dihydrotestosterone (DHT), specific for the androgen receptor, starting before puberty and continuing until adult age (Manneras et al., 2007). We reported that these rats have irregular cycles, polycystic ovaries and display metabolic features including obesity, elevated circulating leptin levels and insulin resistance and thus present a model of DHT-induced PCOS. In addition, they have higher hypothalamic expression of AR mRNA and higher adipose tissue leptin mRNA levels in adulthood (Feng et al., 2009; Manneras et al., 2007; Manneras et al., 2008). In the present study we aimed to identify steps linking androgen signaling to the regulation of affective symptoms in vivo and compared behavioral responses in female rats continuously exposed to DHT from puberty (a model of DHT-induced PCOS) and in rats exposed to DHT for 1 week. Because androgen *and* leptin may be involved in distinct or overlapping behavioral responses in rodents(Leshan et al., 2006; Zuloaga et al., 2008), we investigated the influence of leptin signaling on androgen-dependent regulation of behavior in female rats with DHT-induced PCOS and female rats exposed to DHT for 1 week.

Materials and methods

Animals

Eleven Wistar dams, each with eight to nine female pups, were purchased from Charles River (Sulzfeld, Germany). Pups were raised with a lactating dam until 21 d of age and then housed four to five per cage in an air-conditioned room (21–22 °C) at 55–65% humidity and a 12-h light/12-h dark cycle. Rats had free access to tap water and phytoestrogen-free rodent chow (Teklad global 16% protein rodent diet, 2016, Harland). This study was approved by the Animal Ethics Committee of the University of Gothenburg.

Experimental design and procedure

Experiment 1: rats with DHT-induced PCOS

At 21 d of age, 24 rats were implanted with subcutaneous 90-d continuous-release pellets (Innovative Research of America, Sarasota, FL) containing 7.5 mg of DHT (daily dose, 83 μg) or identical pellets lacking the bioactive molecule ($n=12$ rats/group). Rats with DHT-induced PCOS display typical biochemical, morphological, and metabolic changes of PCOS (Manneras et al., 2007). The dose of DHT we used modulates hypothalamic AR expression in female rats (Feng et al., 2009).

The rats were weighed weekly for 12 weeks after implantation of the pellet. Estrus cyclicity was determined by daily vaginal smears, beginning at 11 weeks of age. Control rats were tested in the estrus phase; DHT-induced PCOS rats were acyclic and tested independent of cycle day. After the behavioral assessments at 15 weeks of age, rats were deeply anesthetized with thiobutabarbital sodium (130 mg/kg i.p.; Inactin, Sigma, St. Louis, MO). All blood samples were obtained by heart puncture. Sera were prepared by clotting and centrifugation and stored at −20 °C until analysis. Dissected tissues were weighed and either immediately frozen in liquid nitrogen and stored at −80 °C for western blotting and hormone analyses or fixed in a 4% formaldehyde neutral-buffered solution for 24 h at 4 °C and embedded in paraffin for histological and immunochemical analyses.

Experiment 2: rats exposed to DHT for 1 week

At 21 d of age, 36 rats were randomly assigned to four groups: vehicle (control), DHT, flutamide, or DHT/flutamide ($n=9$/group). At week 14, rats received daily subcutaneous injections of vehicle (100 μl sesame oil), DHT (1.66 mg/kg/100 μl oil), flutamide (10 mg/kg/100 μl oil), or DHT (1.66 mg/kg/50 μl oil)/flutamide (10 mg/kg/50 μl oil) for 1 week (Feng et al., 2009). The dose of DHT was the same as in Experiment 1, and the dose of flutamide (10 mg/kg/100 μl oil) was decided from a dose–response study ($n=24$) see (Table 1). All drug solutions were freshly prepared before treatment. Estrus cyclicity was determined as in Experiment 1. Control rats were tested in the diestrus phase; DHT exposure disrupted the estrus cycle and DHT-exposed rats were tested independent of cycle day. After the behavioral assessments at 15 weeks of age, rats were euthanized as in Experiment 1.

A flow chart of the experiments is shown in Supplemental Fig. 1.

Behavioral assessment

In both experiments, behavior was assessed at 15 weeks of age. All rats were habituated to the laboratory environment for 1 h before testing each day. There was a 1-day rest between tests. All rats were analyzed in the open-field test, object recognition test, and the elevated plus maze (Hart et al., 2010). All experiments were performed under white light during the light phase of the light/dark cycle, and behavior was recorded with a video camera (Canon MD110, Canon, Japan). The behavioral tests were evaluated by three investigators who were unaware of the group allocation. The order of the tests was designed to minimize potential confounds of multiple tests. The following variables were analyzed: activity (locomotion and rearing), exploration (preference for the safe compartments or risky areas), grooming (scratching, biting, or licking any part of the body), approach-avoidance, and the number of stretch-attends.

Open-field test

An open field device (60 cm × 60 cm × 60 cm) was divided into 16 squares by black lines. The inner four squares (30 cm × 30 cm) were designated as the center and the surrounding squares as the periphery. The illumination at floor level was 60 lx. At the beginning of the test, a rat was removed from the home cage and gently placed in a corner square with the head facing the same corner of the open field. In addition to the locomotor activity (measured by line crossings), we recorded the time spent and number of entries into the center, the number of grooming events, the time spent grooming, the number of rearing events, and the number of line crossings in the center and periphery. After 10 min, the rat was removed and returned to its home cage, and the arena was cleaned with 70% ethanol to remove odors. In this test, the natural tendency of an animal to explore the arena competes with their tendency to avoid the open, brightly lit central area.

Table 1
Dose–response effects of one week of flutamide exposure on body, ovarian, adrenal gland and individual fat weights, and serum and tissue hormone concentrations.

Variable	Vehicle ($n=3$)	DHT (1.66 mg/kg) ($n=3$)	Flutamide			DHT (1.66 mg/kg) /Flutamide		
			10 mg/kg ($n=3$)	50 mg/kg ($n=3$)	100 mg/kg ($n=3$)	10 mg/kg ($n=3$)	50 mg/kg ($n=3$)	100 mg/kg ($n=3$)
Body weight (kg)	0.26 ± 0.01	0.26 ± 0.00	0.27 ± 0.02	0.28 ± 0.01	0.27 ± 0.01	0.27 ± 0.02	0.28 ± 0.01	0.26 ± 0.02
Ovarian weight (mg)	88.87 ± 4.09	58.67 ± 4.63***	79.33 ± 4.06	84.67 ± 1.45	69.67 ± 0.88*	78.33 ± 3.71	66 ± 6.56**	75.33 ± 3.18
Adrenal weight (mg)	64.40 ± 4.26	72.90 ± 5.31	75.73 ± 2.42	94.63 ± 7.67**	86.67 ± 7.28*	78.43 ± 4.45	86.37 ± 4.95*	79.90 ± 1.82
Ovary weight/BW	0.35 ± 0.01	0.22 ± 0.01***	0.29 ± 0.01	0.31 ± 0.01	0.26 ± 0.01**	0.30 ± 0.01	0.24 ± 0.03***	0.29 ± 0.02
Adrenal gland weight/BW	0.25 ± 0.02	0.28 ± 0.02	0.28 ± 0.02	0.34 ± 0.02	0.32 ± 0.02	0.30 ± 0.04	0.31 ± 0.01	0.31 ± 0.03
Serum								
Testosterone (nmol/l)	0.29 ± 0.12	0.32 ± 0.02	0.34 ± 0.12	0.42 ± 0.04	0.28 ± 0.09*	0.47 ± 0.09	0.33 ± 0.03	0.60 ± 0.10
17β-estradiol (pmol/l)	69.33 ± 8.74	59.17 ± 1.20	62.00 ± 5.22	113.67 ± 14.05**	94.83 ± 10.43	79.83 ± 1.69	81.67 ± 2.03	89.17 ± 7.17
Progesterone (nmol/l)	32.77 ± 9.79	40.60 ± 11.91	39.33 ± 11.35	35.48 ± 8.26	34.47 ± 6.86	57.97 ± 13.97	46.07 ± 10.99	52.69 ± 23.07
Ovary								
Testosterone (nmol/mg)	ND	0.0167 ± 0.0033	0.0500 ± 0.0153	0.0567 ± 0.0273	0.0433 ± 0.0203	0.0200 ± 0.0058	0.0367 ± 0.0133	0.0300 ± 0.0000
17β-estradiol (pmol/mg)	ND	70.71 ± 9.86	395.70 ± 118.16	1826.39 ± 460.79***	416.84 ± 134.55	86.18 ± 5.91	180.68 ± 33.28	266.02 ± 58.82
Progesterone (nmol/mg)	ND	2.01 ± 0.17	1.85 ± 0.76	2.12 ± 0.34	1.18 ± 0.33	2.66 ± 0.68	2.04 ± 0.29	1.71 ± 0.32

Values are mean ± SEM. BW, body weight; ND, not determined.
Multiple comparisons between data were performed with one-way ANOVA and Dunnett's post hoc test. $p<0.05$ was considered statistically significant.
*** $p<0.001$ vs. vehicle.
* $p<0.05$ vs. vehicle.
** $p<0.01$ vs. vehicle.

Object recognition test

Next, object recognition memory was assessed. Trials consisted of a sample trial (T1) and a recognition trial (T2), conducted 2 h apart. In T1, a rat was placed in the open field, which contained two identical objects (a white wood cylinder and a green wood pyramid). The rat was allowed to explore the objects undisturbed for 10 min. Exploration was defined as the animal having its head within 2 cm of the object while looking, sniffing, or touching it with the nose. In T2, one copy of the familiar object and a novel object was placed in the same location as in T1. The rat was placed in the open field for 5 min. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preference for particular locations or objects. The time spent exploring the familiar and novel object was recorded. After each rat was tested, the objects were cleaned with 70% ethanol to remove odors.

Elevated plus maze

The movement of rats was recorded in an elevated plus maze consisting of two open arms and two closed arms (50 × 10 cm). The closed arms (safe areas) were enclosed by 38-cm-high plexiglass black walls; the open arms (risky areas) had transparent plastic ledges (0.3 cm) to prevent falls. The maze was made from dark PVC and placed 73 cm above the floor in a silent, dimly illuminated room (3 × 4 m; the light intensity was 100 lx in the open and 60 lx in the closed arms). A rat was placed directly in intersection of the open and closed arms and videotaped for 10 min. Entries into the closed and open arms were counted and expressed as percentage of the total number of entries. Placement of both forepaws on an arm was considered an entry. We also measured the time spent on the open arms (expressed as the percentage of the total time spent in all arms) and the time spent grooming, as well as the number of partial entries and stretched-attends in open and closed arms. The maze was cleaned with alcohol after each rat was tested.

Brain morphology analysis and microscopy

Six rats with DHT-induced PCOS and three rats exposed to DHT for 1 week were perfused for immunohistochemical analyses. Rats were deeply anesthetized and perfused via the left ventricle with 200 ml of cold 0.9% sodium chloride followed by 100 ml Histofix (Histolab, Sweden). Brains were dissected, postfixed in Histofix containing 20% sucrose for 24 h at 4 °C, and incubated in 0.1 M PBS containing 30% sucrose for at least 48 h at 4 °C. Serially frozen frontal sections (20 μm) were cut and stored in tissue culture wells containing 30% sucrose and 30% ethylene glycol in 0.1 M PBS (pH 7.4) at −20 °C. After immunohistochemical staining, we used a previously described technique (Feng et al., 2009, 2010). Free-floating sections underwent three five-min washes in Tris-buffered saline (TBS; 50 mM Tris, 0.9% NaCl, pH 7.5). To eliminate endogenous peroxidase and nonspecific binding, sections were incubated with 3% H_2O_2 for 30 min, 0.5% Triton X-100 for 10 min at room temperature and with 10% normal horse serum for 1 h at 37 °C. Next, sections were incubated with primary antibody [rabbit anti-AR (sc-816), 1:200; rabbit anti-leptin (Ob, sc-842), 1:100, Santa Cruz Biotechnology, Santa Cruz, CA)] for 1 h at 37 °C and then overnight at 4 °C. After washing, sections were incubated with biotinylated secondary antibody for 1 h at 37 °C. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for the avidin-biotinylated peroxidase complex detection system according to the manufacturer's instructions. Antigens were visualized by reaction with the chromogen 3,3′-diaminobenzidine-tetrahydrocholoride (Sigma) and hydrogen peroxidase for 1 min. Sections were imaged on an Olympus DP50 microscope (Japan) under bright-field optics and photographed with Image-Pro plus software (version 5.0, Media Cybernetics, Bethesda, MD). In additional control studies of the specificities of primary antibodies, we used a four-point scale (Feng et al., 2009) to score the relative number of AR- and leptin-positive cells: −, none; +, 1–10; ++, 11–100; and +++, >100. Variability between immunohistochemical replicates was controlled by having an equal number of animals for each group in each immunohistochemical run. Brain regions were identified as described (Paxinos and Watson, 2009). Quantification was performed by two observers blinded to the hormonal states of the rats.

Quantitative western blot analysis

Western blot analysis was performed as described (Shao et al., 2007). Equal amounts of protein were directly electrophoresed on 4–12% one-dimensional Bis–Tris gels (Novex, San Diego, CA). In experiments examining AR expression, the membrane was cut into two pieces along a line corresponding to approximately 61 kDa. The

section containing higher molecular weights was exposed to antibodies against the AR (sc-816, 1:250) or phosphorylated signal transducer and activator of transcription 3 antibody [phosphor-STAT3 (Tyr705), 1:500, Cell Signaling Technology, Danvers, MA]. The lower molecular weight section was exposed to α-tubulin antibody (B-5-1-2, 1:1000, Sigma, St. Louis, MO) as a loading control. The gels were stained with Coomassie blue to confirm equal loading. The membranes were washed three times for 5 min each in TBS-0.05% Tween 20 and incubated with the appropriate alkaline phosphatase-linked secondary antibodies (Bedford, MA) with 1:40,000 or 1:80,000 dilutions for 2 h. The immunosignal-CDP-Star substrate for alkaline phosphatase system (Tropix, Bedford, MA) was used to visualize protein bands. Immunoblot signals were photographed with a cooled charge-coupled device camera (LAS-1000, Fujifilm, Tokyo, Japan). Individual bands were quantified directly from the membranes by densitometry with ImageQuant (version 5.0) software (Molecular Dynamics, Sunnyvale, CA).

Determination of endocrine hormone and soluble leptin receptor concentrations

Levels of testosterone, 17β-estradiol, and progesterone in serum, adipose tissue, and brain were determined with enzyme-linked immunoassay kits (R050-201, R056-101 and R066-101, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) as recommended by the manufacturer. Leptin levels in the mesentery, hippocampus, and hypothalamus were determined in homogenized tissues as described (Feng et al., 2010). Levels of leptin (EZRL-83 K, Linco Research, St. Charles, MO) and soluble leptin receptor (sObR, E90083Ra, USCN Life Science, Wuhan, China) in serum and tissues were measured with enzyme-linked immunosorbent assays. Protein concentrations in homogenates were measured with the Bradford method as described (Shao et al., 2007). Circulating corticosterone levels were determined with enzyme-linked immunoassay kits (900–097, Assay Designs, Ann Arbor, MI) as recommended by the manufacturer.

Data analysis and statistics

All data are presented as mean ± SEM. Multiple comparisons were analyzed for statistical significance by one-way ANOVA and Dunnett's post hoc test (SPSS, version 18.0; Chicago, IL); $p<0.05$ was considered statistically significant.

Results

Rats with DHT-induced PCOS are obese, insulin resistant, have high circulating leptin concentrations and display a PCO-like ovarian morphology and disrupted estrus cyclicity (Manneras et al., 2007; Manneras et al., 2009). To determine whether the flutamide dose was sufficient to modulate effects on 1 week of DHT exposure on behavioral performance without interfering with estrogen and progesterone in rats, a dose–response study ($n=24$) was performed to evaluate the antagonistic properties of flutamide on ovarian hormone synthesis and body, ovarian, adrenal gland, and fat weights (Table 1). The dose 10 mg/kg of flutamide was selected since it did not affect body, ovarian or adrenal weights, and sex steroid profile (Table 1).

Rats with DHT-induced PCOS display anxiety-like behavioral changes

In the open field test, rats with DHT-induced PCOS had fewer line crossing in the periphery and center (Fig. 1A) than controls, and fewer rearing events in the periphery (Fig. 1B). However, there were no differences in time spent in the center or periphery (Fig. 1A). In the elevated plus maze, rats with DHT-induced PCOS made fewer entries into either open or closed arms than controls and spent less time spent in the open arms (Fig. 1C). There were no differences in the number and time of grooming events (Fig. 1D) or the number of stretch-attends (Fig. 1E). In the object recognition test, PCOS rats and controls did not differ in the time spent examining familiar and novel objects (Fig. 1F).

One week of DHT induces behavioral changes

In the open field test, rats exposed to DHT for 1 week had fewer line crossings in the periphery and center, indicating less overall locomotor activity, than controls (Fig. 2A). Neither flutamide nor DHT/flutamide affected the number of line crossings or the time and number of rearing events in the center or periphery (Figs. 2A and B). In the elevated plus maze, there were no difference between groups in the number of entries or time spent in open and closed arms (Fig. 2C), the number and duration of grooming events (Fig. 2D), or the number of stretch-attends (Fig. 2E). In the object recognition test, rats exposed to DHT and DHT/flutamide did not differ in the time spent examining familiar and novel objects (Fig. 2F).

Effects of DHT exposure on body and tissue weight, circulating sex steroids and corticosterone

Body weight development, ovarian weight, circulating steroids and corticosterone levels, and hypothalamic expression of corticotrophin-releasing hormone have been reported in rats with DHT-induced PCOS (Feng et al., 2009; Johansson et al., 2010; Manneras et al., 2007, 2008, 2009).

Body weight, including fat depots, and ovarian weights, and circulating steroid hormone in rats exposed to DHT and/or flutamide for 1 week are shown in Table 2. Although ovarian weight was significantly decreased after DHT exposure, circulating testosterone, 17β-estradiol, and progesterone levels were unaltered in all groups. Aberrant secretion of glucocorticoids has been implicated in major depression (Steckler et al., 1999). One week of DHT exposure did not affect circulating corticosterone levels.

DHT exposure affects AR expression in the brain in a region-specific manner

The density of AR-positive cells in specific hypothalamic regions, including the paraventricular hypothalamic nucleus (PVN), medial preoptic area (MPO), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), and premammillary nucleus (PMV) from Experiments 1 and 2 is presented in (Table 3).

Previously we have demonstrated that hypothalamic AR protein expression is higher in DHT-induced PCOS rats and that AR-positive cells are increased in MPO (Feng et al., 2009). Here we present a similar pattern in the MPO, while no major differences in the number of AR-positive cells are present on other regions between rats with DHT-induced PCOS and controls (Table 3).

Examination of AR expression in the brains of rats exposed to DHT and/or flutamide for 1 week revealed a different expression pattern of AR immunostaining compared to DHT-induced PCOS rats (Table 3). The density and number of AR-positive cells were lower in the retrosplenial agranular (RSA), arcuate nucleus (ARC), and nucleus of the solitary tract (Sol) in rats exposed to DHT for 1 week (Figs. 3A, C, and D). One week of flutamide and DHT/flutamide maintained AR immunoreactivity in the RSA, but not the Sol (Figs. 3A and D), and increased the number of AR-positive cells in the VMH, DMH, ARC, and PMV (Table 3), but did not change AR expression in the hippocampus, including the CA2 region (Fig. 3B). Western blot analysis revealed significant lower hypothalamic AR expression after 1 week of DHT exposure than in controls, but not after flutamide or DHT/flutamide exposure (Fig. 3E).

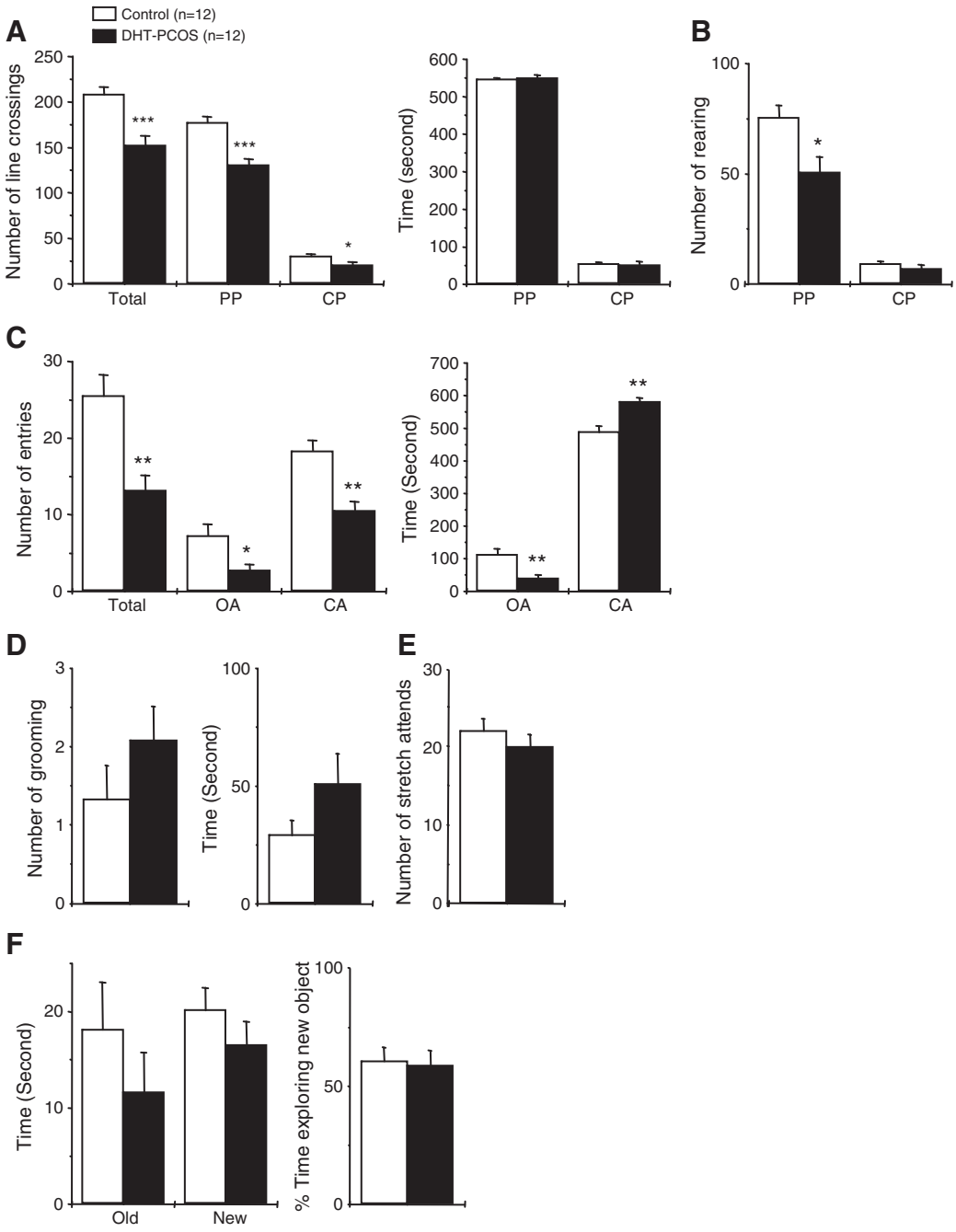

Fig. 1. Rats with DHT-induced PCOS display anxiety-like behavior but not cognitive impairment. (A) Number of line crossings and time spent and (B) number of rearings in the peripheral part (PP) and central part (CP) of the open field. Rats with DHT-induced PCOS exposure had significantly fewer line crossings and rearings. (C) Number of entries and time spent in open arms (OA) and closed arms (CA) of the elevated plus maze. Rats with DHT-induced PCOS spent less time in the open arms and more time in the closed arms than controls. (D) Number of groomings and time spent on grooming and (E) number of stretch-attends in the elevated plus maze. (F) Time spent exploring familiar and novel objects in the object recognition test. Values are mean ± SEM ($n = 12$ rats/group). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. vehicle.

DHT-induced PCOS or 1 week of DHT activates leptin signaling in adipose tissues

Rats with DHT-induced PCOS model become obese, with increased weight of inguinal and parametrial adipose tissue (Johansson et al., 2010; Manneras et al., 2007, 2008) and elevated levels of leptin in the circulation (Manneras et al., 2007) and of leptin mRNA expression in mesenteric adipose tissue (Manneras et al., 2008).

One week of DHT exposure increased inguinal and parametrial fat depots (Table 2). Circulating leptin levels were marginally affected by 1 week of DHT exposure, but circulating and adipose tissue leptin levels were increased in rats exposed to flutamide and DHT/flutamide (Table 2). Because leptin induces tyrosine phosphorylation of STAT3, a key downstream mediator of ObR signaling (Fan et al., 2008), we characterized the expression of phospho-STAT3 (Tyr705) in rats exposed to DHT or DHT/flutamide for 1 week. DHT decreased phospho-STAT3 expression in mesenteric adipose tissue, as shown by western blot analysis (Fig. 4F). There was a trend toward decreased phospho-STAT3 expression in rats exposed to DHT/flutamide for 1 week (Fig. 4F). We hypothesized that DHT-induced AR-dependent regulation of leptin synthesis and ObR activation in adipose tissues. However, western blot analysis did not reveal any changes in adipose tissue AR expression in rats exposed to DHT and/or flutamide for 1 week (Fig. 3F).

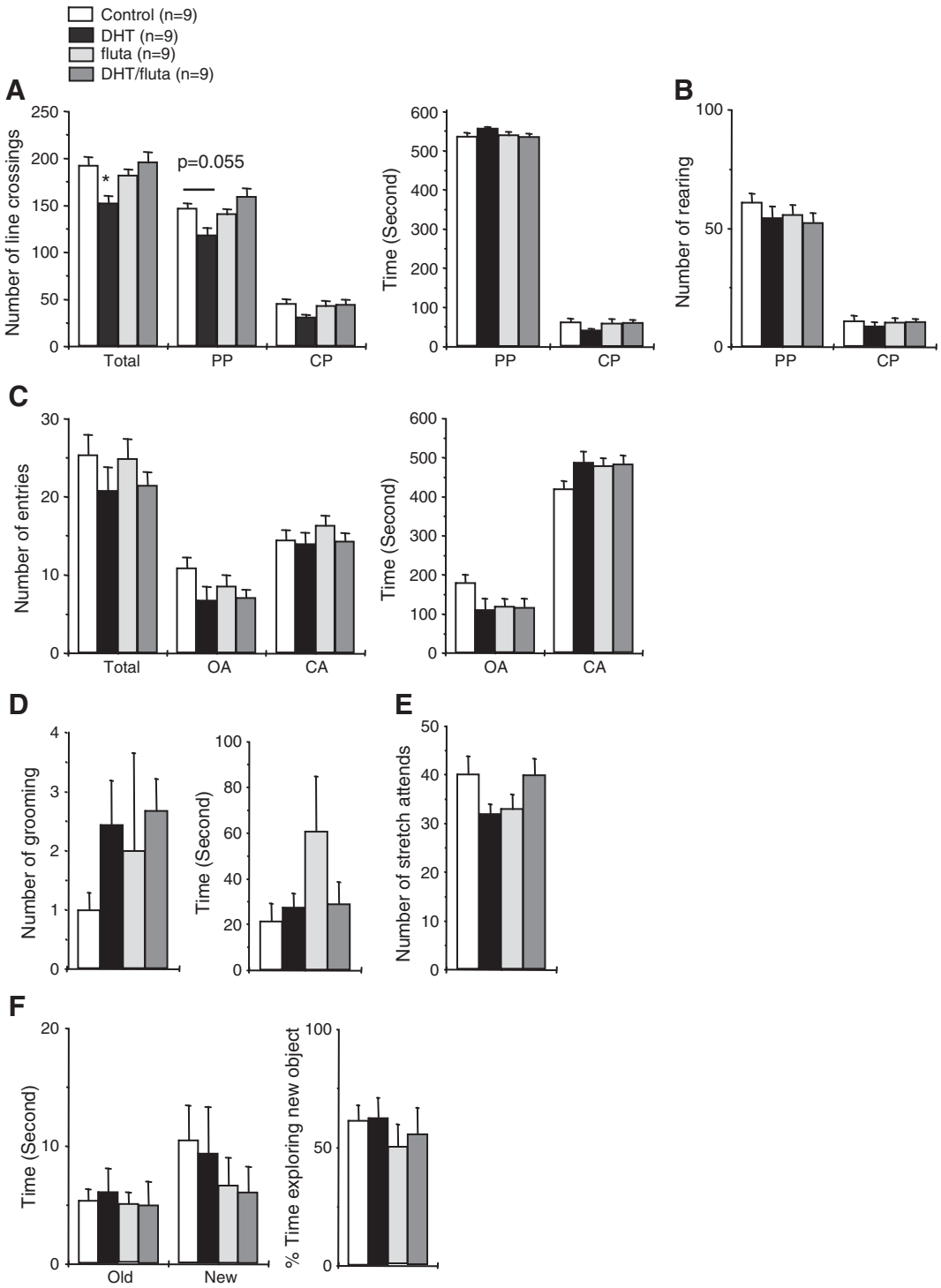

Fig. 2. One week of DHT and/or flutamide induces behavioral changes in female rats. Rats received daily subcutaneous injection of vehicle, DHT, flutamide, or DHT/flutamide for 1 week. (A) Number of line crossings and time spent and (B) number of rearing in the peripheral part (PP) and central part (CP) of the open field. Rats that received DHT for 1 week had significantly smaller number of total line crossings (PP + CP). (C) Number of entries and time spent in the open arms (OA) and closed arms (CA) in the elevated plus maze. (D) Number of groomings and time spent grooming and (E) number of stretch-attends in the elevated plus maze. (F) Time spent exploring familiar and novels objects in the object recognition test. Values are mean ± SEM ($n = 9$ rats/group). $^{*}p<0.05$ vs. vehicle.

DHT-induced PCOS or 1 week of DHT stimulates uptake of leptin and expression of soluble leptin receptor in the brain

Leptin was expressed in cortex, hippocampus, and hypothalamus in rats with DHT-induced PCOS. The density and number of leptin-positive cells were lower in the hippocampus (dentate gyrus) and MPO in DHT-induced PCOS rats than in controls, but not in other hypothalamic nuclei (Supplemental Fig. 2, Table 4).

The density and number of leptin-positive cells were lower in the RSA, hippocampus, and MPO of rats exposed to DHT for 1 week than in controls (Fig. 4, Table 4). Interestingly, leptin levels in the hypothalamus, but not in the hippocampus, were higher in rats exposed to DHT for 1 week (Table 2). In addition, leptin levels in the hypothalamus were lower in the DHT/flutamide group (Table 2). The pattern of leptin immunoreactivity (Fig. 4, Table 4) in the ARC was similar to that of AR immunoreactivity in rats exposed to DHT or DHT/flutamide for

Table 2
Effects of 1 week of DHT and/or flutamide exposure on body, ovarian, and fat depot weights, serum and tissue hormone concentrations, and other parameters.

Variable	Vehicle	DHT	Flutamide	DHT/flutamide
Body weight (kg)	0.24 ± 0.01	0.26 ± 0.01	0.24 ± 0.01	0.25 ± 0.01
Ovary weight (mg)	87.52 ± 3.36	64.55 ± 1.48***	81.47 ± 2.15	88 ± 4.88
Fat depot weights				
Mesenteric (g)	1.45 ± 0.06	1.80 ± 0.13	1.70 ± 0.06	1.57 ± 0.14
Inguinal (g)	0.95 ± 0.08	1.64 ± 0.16***	1.07 ± 0.06	0.80 ± 0.05
Parametrial (g)	2.86 ± 0.29	4.33 ± 0.42**	3.36 ± 0.07	3.34 ± 0.30
Fat depots/BW				
Mesenteric (g/kg)	5.95 ± 0.19	6.83 ± 0.44	6.92 ± 0.15	6.24 ± 0.61
Inguinal (g/kg)	3.90 ± 0.34	6.20 ± 0.52**	4.37 ± 0.28	3.19 ± 0.24
Parametrial (g/kg)	11.66 ± 1.00	16.48 ± 1.69	13.79 ± 0.54	13.22 ± 1.22
Steroid hormones				
Testosterone (nmol/l)	0.38 ± 0.15	0.22 ± 0.03	0.58 ± 0.06	0.54 ± 0.08
17β-estradiol (pmol/l)	80.00 ± 13.33	77.25 ± 3.79	78.25 ± 4.62	78.67 ± 5.43
Progesterone (nmol/l)	22.99 ± 6.24	19.51 ± 3.80	85.27 ± 35.94	28.28 ± 9.51#
Leptin				
Serum (ng/ml)	4.47 ± 0.52	5.72 ± 0.58	6.66 ± 0.40*	6.41 ± 0.61*
Adipose (mesenteric, pg/mg)	675.51 ± 28.24	557.16 ± 11.44	1029.39 ± 80.85***	615.77 ± 44.75
Hippocampus (pg/mg)	79.81 ± 4.02	79.86 ± 1.76	71.19 ± 4.3	72.34 ± 2.64
Hypothalamus (pg/mg)	67.81 ± 2.44	143.76 ± 13.21***	91.89 ± 2.5	91.97 ± 3.76#
Soluble leptin receptor (sObR)				
Adipose (mesenteric, pg/ml)	7331.76 ± 706.33	6480.29 ± 331.95	7399.10 ± 409.28	4736.54 ± 259.36**
Hippocampus (pg/ml)	1877.89 ± 277.96	1282.49 ± 115.32	1886.06 ± 304.65	1694.77 ± 161.88
Hypothalamus (pg/ml)	5044.45 ± 396.43	9885.72 ± 1094.30**	5893.10 ± 1109.33	5564.26 ± 820.93
Corticosterone (ng/ml)	110.99 ± 39.68	106.32 ± 29.41	169.73 ± 42.73	173.07 ± 42.17

Values are mean ± SEM; $n = 6$ per treatment group. BW, body weight.
*** $p<0.001$ vs. vehicle.
** $p<0.01$ vs. vehicle.
* $p<0.05$ vs. vehicle.
# $p<0.05$ vs. DHT.

1 week (Fig. 3, Table 3). In addition, flutamide or DHT/flutamide exposure did not alter the number of leptin-positive cells in hypothalamic nuclei, including the PVN, VMH, DMH, and PMV (Table 4). Neither DHT nor flutamide affected phospho-STAT3 protein levels, as shown by western blot (Fig. 4E). Surprisingly, 1 week of DHT increased the levels of soluble ObR in the hypothalamus, but not in the hippocampus, by roughly 50% (Table 2).

Discussion

The relationship between different regions of the brain and specific behaviors is unclear (Leshan et al., 2006). In this study, we examined behavioral traits and possible mechanism of action of the AR and ObR/leptin in a rat model of DHT-induced PCOS and in rats exposed to DHT and/or flutamide for 1 week. Our findings suggest that an interaction of peripheral and central androgen and leptin signaling affects the neural mechanisms of behavior in female rats. Future studies are warranted to address how the two signals exert their effect on behaviors.

Rats with DHT-induced PCOS displayed anxiety-related behaviors, including decreased locomotion, rearing, and exploration, consistent with clinical observations in previous studies of women with PCOS (Deeks et al., 2010; Jedel et al., 2010b). In these studies, symptoms of anxiety were more prominent than symptoms of depression. Locomotor activity was also decreased in rats exposed to DHT for 1 week, albeit to a lesser extent than in rats with DHT-induced PCOS. This difference may reflect differences in the duration of DHT exposure (1 week vs. 90 days). Rats were at the same age when the experiments were performed and received the same dose of DHT. Concomitant flutamide exposure blocked the adverse changes in locomotor activity, suggesting that AR signaling pathways participate in the locomotor response in female rats. The different responses

Table 3
AR immunoreactivity in selective brain regions in rats with DHT-induced PCOS (Experiment 1) and in rats exposed to DHT and/or flutamide for 1 week (Experiment 2).

Brain region	Experiment 1		Experiment 2			
	Control ($n=6$)	DHT-PCOS ($n=6$)	Vehicle ($n=3$)	DHT ($n=3$)	Flutamide ($n=3$)	DHT/fluta ($n=3$)
Cortex	+/++	+/++	+/++	+	+	++
Hippocampus						
CA1, CA2, CA3	−	−	−	−	−	−
Hypothalamus						
PVN	+	+	+	−	−	−
MPO	+	+++	+	−	−	−/+
VMH	+/++	+	++	−	−	+/++
DMH	+	+	+	−	−	−/++
ARC	+/++	++	+/++	−	++/+++	++/+++
ME	+/++	−/+	+/++	+	+	++
PMV	++	+/++	++	−	++/+++	++/+++

Immunoreactivity was scored according to the number of immunoreactive cells: −, none; +, 1–10 cells; ++, 11–100 cells; +++, >100 cells.
PVN, paraventricular hypothalamic nucleus; MPO, medial preoptic area; VMH, ventromedial hypothalamus nucleus; DMH, dorsomedial hypothalamic nucleus; ARC, arcuate nucleus; ME, median eminence; PMV, premammillary nucleus, ventral part.

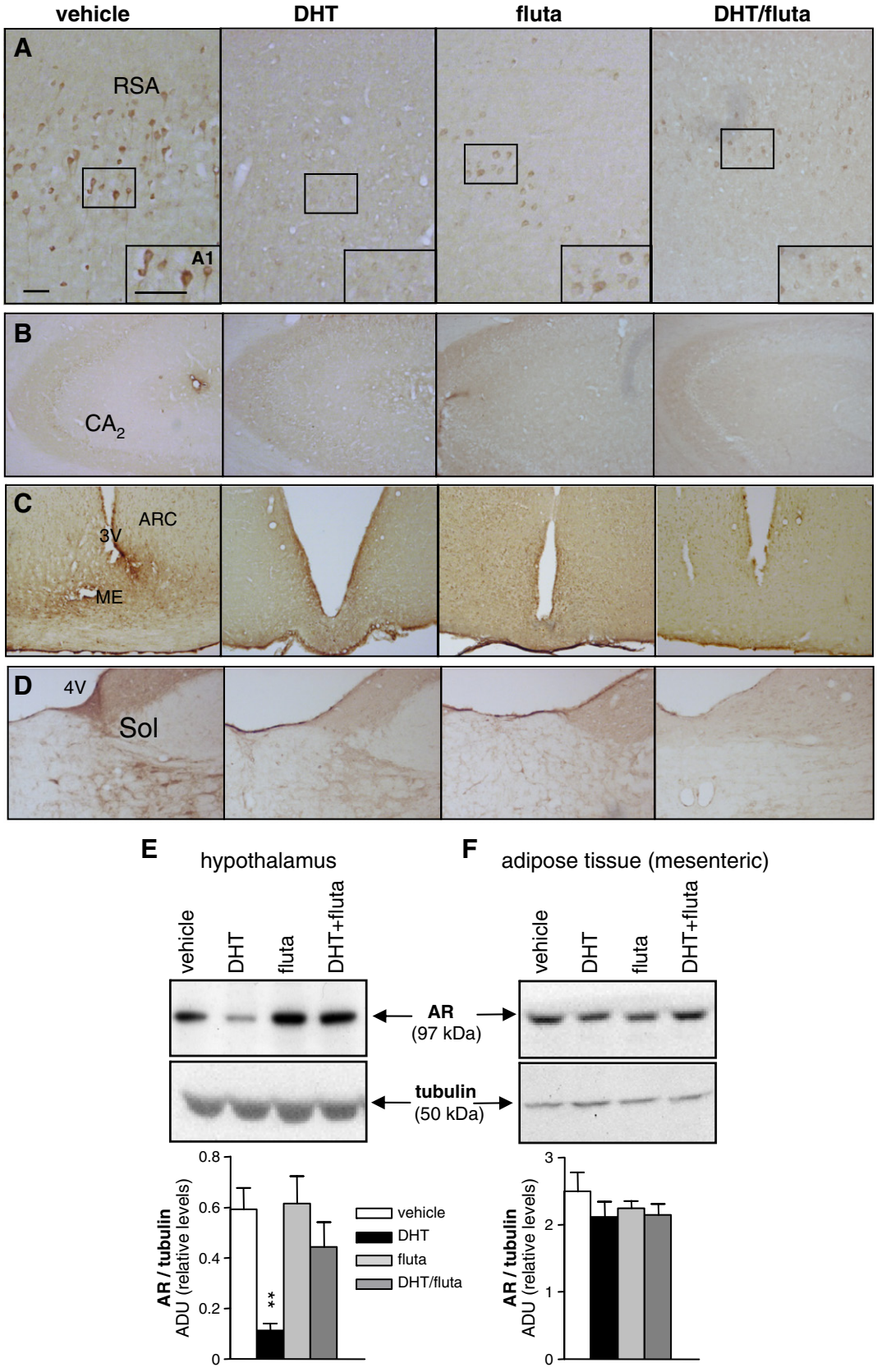

Fig. 3. Brain region-specific and tissue-specific regulation of AR expression in rats exposed to DHT and/or flutamide for 1 week. A–D, Immunohistochemical staining for AR in (A) cerebral cortex, (B) hippocampus CA2, (C) hypothalamus, and (D) nucleus of the solitary tract (Sol) after 1 week of treatment with vehicle, DHT, flutamide, or DHT/flutamide. DHT decreased the number of AR-immunoreactive cells in the cerebral cortex, hippocampus CA2, and hypothalamic ARC. Images in A1 are at higher magnifications than A. Data are representative of at least two independent experiments with similar results ($n=3$ rats/group). Scale bars, 100 μm. RSA, retrosplenial agranular; ARC, arcuate nucleus; ME, median eminence; 3v, third ventricle; 4v, fourth ventricle. E and F, Western blot analysis of AR and α-tubulin expression in the hypothalamus (E) and mesenteric adipose tissue (F). Equal amount of proteins was separated on 4–12% one-dimensional Bis–Tris gels and immunoblotted with antibodies against AR or α-tubulin. Band intensities were quantified by densitometry. Data are expressed as arbitrary densitometric units (ADU). Values are mean ± SEM ($n=6$ rats/group). $^{**}p<0.01$ vs. vehicle.

suggest that multiple mechanisms are involved in the modulation of specific brain regions, which ultimately influence behavioral function. However, these findings need to be further explored.

Involvement of AR in regulation of behavior in females

Although no molecular mechanism has been established for central androgen-mediated behavior in females, androgen–AR signaling is thought to be important in regulating behavior in DHT-treated male mice (Raskin et al., 2009). The female brain is exposed to significantly lower levels of testosterone (Weisz and Ward, 1980), and we and others have demonstrated that the AR is spatiotemporally expressed in the adult female rat brain during the estrous cycle (Feng et al., 2010; Handa et al., 2008). In this study, we demonstrated regional regulation of AR expression in rats with DHT-induced PCOS and in female rats exposed to DHT for 1 week. One week of DHT decreased the density and number of AR-positive cells, consistent with a previous report (Feng et al., 2010). Further, 1 week of flutamide or DHT/flutamide exposure affected certain regions of the brain (e.g., hypothalamic ARC and PMV). On the other hand, in a previous study, the AR was expressed at a higher level in the hypothalamic MPO in rats with DHT-induced PCOS than in controls (Feng et al., 2009), and in the current study we found a similar pattern in MPO compared to controls but not in other hypothalamic regions. In considering these divergent results (1 week vs. 90 days), again the difference may simply reflect differences in the duration of DHT exposure. DHT is a non-aromatizable androgen. Although DHT's metabolite, 5α-androstane-3β, 17β-diol (3β-Adiol) has been shown to function as an endogenous ERβ ligand that could facilitate ER-mediated transcription through classical ER signaling pathways in neuronal cells, selective activation of ERβ has been shown to decrease anxiety-like behaviors, whereas selective activation of ERα has been shown to increase these behaviors (Lund et al., 2005; Walf et al., 2008; Walf and Frye, 2005). Therefore, DHT-induced anxiety-like behavioral changes are likely mediated through ARs in our study.

AR and ObR/leptin interaction

Neurobehavioral deficits in *db*/*db* mice (Sharma et al., 2010) have suggested that hypothalamic leptin signaling is required for normal neuroendocrine function and behavioral responses (Obici, 2009). Because of the anatomic relationship between the AR and leptin signaling pathways in male mouse hypothalamus (Fan et al., 2008), we hypothesized that adipocyte-derived leptin biosynthesis and release interact with DHT in female rat brains. To explore this possibility, we performed an immunohistochemical analysis to determine whether central leptin levels were affected by exposure to DHT and/or flutamide for 1 week. In both groups, the distribution of AR immunoreactivity in the ARC was similar to that of leptin immunoreactivity in rats exposed to DHT and/or flutamide for 1 week. This is in line with previous studies demonstrating that the ObR is expressed at high levels in the hypothalamic ARC (Bjorbaek, 2009), a key site for the leptin-mediated locomotor activity (Coppari et al., 2005). In addition, neonatal androgen exposure decreases leptin responsiveness (leptin induced pSTAT3 or anorexigenic action) which further supports the present findings (Nohara et al., 2011).

Leptin is synthesized and secreted predominantly by adipocytes (Myers et al., 2008). One week of DHT exposure inhibited ObR-specific STAT3 signaling in adipose tissues. Therefore, the peripheral actions of leptin-ObR might contribute to the central androgen-induced behavioral changes. Genetic ablation of the AR blocks leptin-mediated STAT3 transactivation and nuclear translocation in male mouse hypothalamus (Fan et al., 2008). Although we detected increases in hypothalamic leptin levels in the DHT group and a decrease in the DHT/flutamide group, and a trend of increase in circulating leptin concentrations, neither DHT nor flutamide regulated phospho-STAT3 protein expression in the hypothalamus.

In several studies, the central action of leptin resulted in positive regulation of locomotor activity in mice (Coppari et al., 2005; Huo et al., 2009). We studied only the effects of DHT on chronic anxiety-related behavioral responses, not the effects of leptin. However, there is reason to speculate that increases in hypothalamic leptin levels in rats exposed to DHT for 1 week limit the anxiety-related behavioral response, presumably as a consequence of abrogation of the ligand-induced AR action.

Importantly, our findings reveal a complex regulatory role for leptin–ObR signaling in the brain. Although ObR isoforms have been identified in rodent brains (Elmquist et al., 1998; Fei et al., 1997), soluble ObR, a leptin-binding protein, abolishes leptin activity (Myers et al., 2008). It appears that access of soluble ObR to the brain is differentially controlled by androgenic stimulation. The hypothalamic soluble ObR level was significantly higher in rats exposed to DHT for 1 week than in controls. Attenuation of leptin activity by elevated levels of soluble ObR in the hypothalamus after 1 week of DHT exposure may contribute to the DHT-AR-induced anxiety-related behavioral responses. The absence of significant regulation of phospho-STAT3 protein expression in the hypothalamus of DHT-exposed rats may reflect impaired leptin action. Nevertheless, in addition to the central effects of DHT, the increased soluble ObR in the hypothalamus of female rats may argue for a positive effect of increased leptin action on locomotor activity.

AR and hypothalamus–pituitary–adrenal axis interaction

It is well known that corticosterone presents circadian rhythms resulting in changes in the response to the different test used to discriminate anxiety and depression. In the present study, 1 week of DHT exposure did not affect circulating corticosterone concentrations. We have previously demonstrated that continuous exposure of DHT to female rats decreases circulating corticosterone concentrations (control, 845 ± 210 ng/ml vs. PCOS, 253 ± 25 ng/ml; $p < 0.001$) (Manneras et al., 2008). This is in line with previous reports which demonstrate that testosterone inhibits ACTH and corticosterone response to stress (Handa et al., 2009). Therefore, we do not believe that it is altered HPA axis activity that contributes to the anxiety like behavior in these experiments. However, it can't be excluded that DHT modifies hypothalamic neurotransmitters, such as dopamine and serotonin, which clearly modifies behavior.

Clinical interpretations

Comparisons of behavioral changes in rats with DHT-induced PCOS and rats exposed to DHT for 1 week are difficult to interpret because little is known about the time-course of anxiety-related behaviors in rats with DHT-induced PCOS. However, these rats display anxiety-like behavior similar to that in women with PCOS (Benson et al., 2009; Deeks et al., 2010; Jedel et al., 2010b). Symptoms of anxiety and depression in women with PCOS are a significant health concern (Deeks et al., 2010; Jedel et al., 2010b), and the mechanism and appropriate medication are still elusive. Anxiety-related behaviors likely reflect multiple causal factors. In several clinical studies, women with PCOS had reduced quality of life and negative psychological function (Barnard et al., 2007; Coffey et al., 2006; Elsenbruch et al., 2003). Approximately 50% of women with PCOS are obese and resistant to multiple signaling factors, including leptin (Azziz et al., 2004). The higher body mass index and waist-to-hip ratio (Adali et al., 2008) in these women are also associated with increased anxiety (Mansson et al., 2008). We have previously demonstrated that women with PCOS have high circulating sex steroid precursors, androgens and glucuronidated androgen metabolites, as measured with the highly sensitive mass spectrometry, and differ from controls

matched for age, weight and BMI (Stener-Victorin et al., 2010), and that lower free testosterone concentrations are associated with higher symptoms of depression (Jedel et al., 2011). Although lower rather than higher free testosterone was associated with symptoms of depression in PCOS, the circulating concentrations were higher than in women without PCOS (Jedel et al., 2011). Thus we favor the

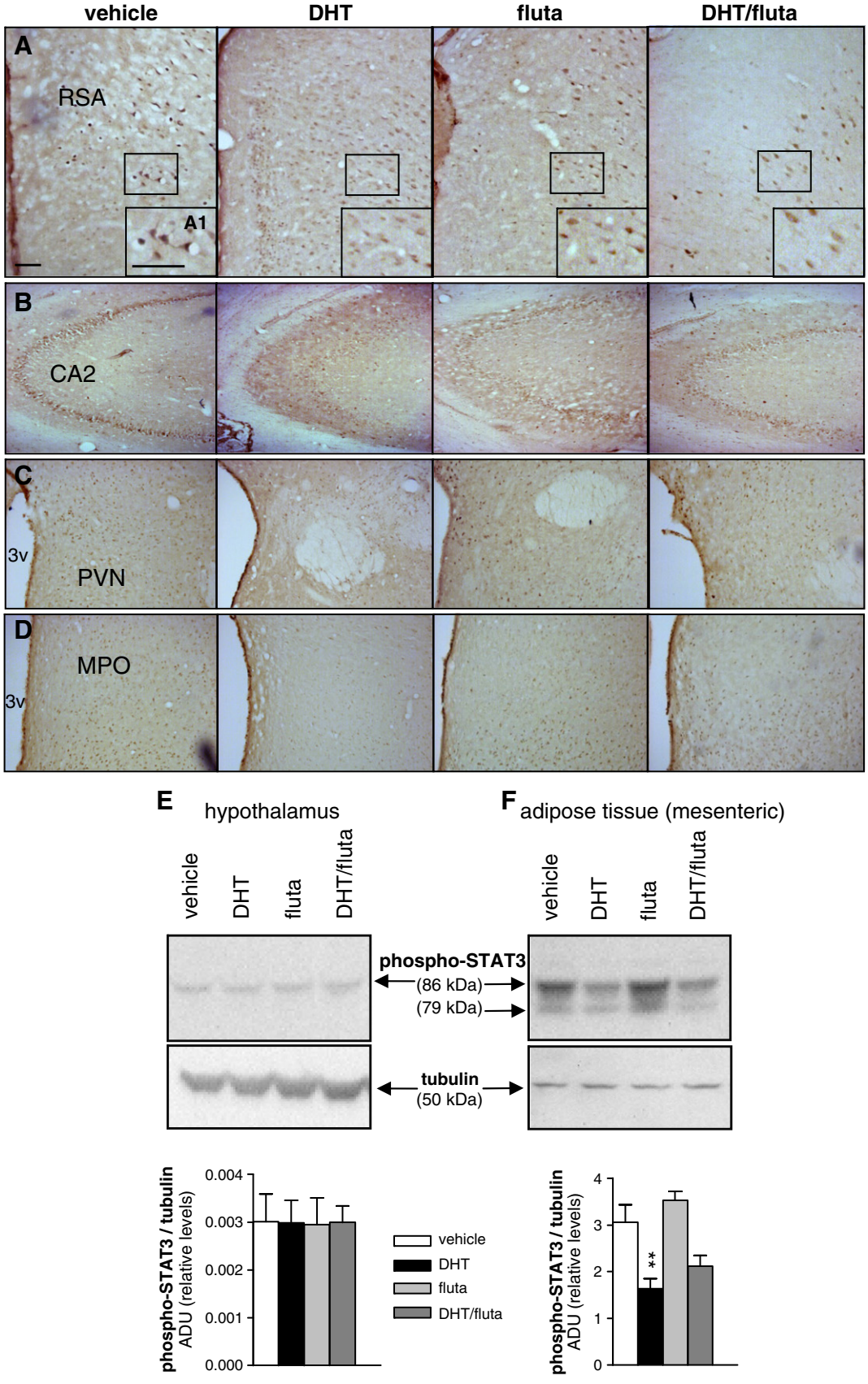

Table 4
Distribution of leptin immunoreactivity in selective brain regions in rats exposed to DHT and/or flutamide for 1 week and rats with DHT-induced PCOS.

Brain region	Experiment 1		Experiment 2			
	Control ($n=3$)	DHT-PCOS ($n=6$)	Vehicle ($n=3$)	DHT ($n=3$)	Flutamide ($n=3$)	DHT/Flutamide ($n=3$)
Cortex	++/+++	++/+++	++/+++	++/+++	++/+++	++/+++
Hippocampus						
CA1, CA2, CA3	++	+	++	−/+	+/++	++
Hypothalamus						
PVN	++	++/+++	++	+/++	++	++
MPO	++	+/++	++	−/+	+/++	+/++
VMH	++	++	++	++	++	++
DMH	++	+/++	++	++	++	+/++
ARC	++	++	++	++	++/+++	++/+++
ME	++	+/++	++	++	++	++/+++
PMV	++/+++	+++	++/+++	+++	+++	+++

Immunoreactivity was scored according to the number of immunoreactive cells: −, none; +, 1–10 cells; ++, 11–100 cells; +++, >100 cells.
PVN, paraventricular hypothalamic nucleus; MPO, medial preoptic area; VMH, ventromedial hypothalamus nucleus; DMH, dorsomedial hypothalamic nucleus; ARC, arcuate nucleus; ME, median eminence; PMV, premammillary nucleus, ventral part.

hypothesis that the androgen signaling is the major contributor to the anxiety-related behavioral responses we observed, and PCOS-related behavioral changes in females might be caused all or in part by other mechanisms such as leptin.

In conclusion, this study revealed anxiety-related behavior in rats with DHT-induced PCOS and in rats exposed to DHT for 1 week. Our results highlight the central role of androgens in behavioral function in female rats and suggest that androgens directly regulate the AR by decreasing its hypothalamic expression. Androgens also increase leptin synthesis in adipocytes, which drives central leptin signaling, and may regulate anxiety-related behaviors. Elucidating mechanisms by which androgens modulate female anxiety-like behavior may uncover useful approaches for treating women with PCOS who have symptoms of anxiety.

Author contributions

Conceived and designed the experiments: YF, RS, HB, ESV. Performed the experiment: YF, RS, BW, TW, JJ, SS, WW, EE, ESV. Analyzed the data: YF, RS, TW, WW, SS, EE, ESV. Contributed reagents/materials/analysis tools: RS, JJ, EE, HB, ESV. Interpreted the data: YF, RS, HB, ESV. Wrote the paper: YF, RS, ESV. Assisted in editing the manuscript: HB.

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Fig. 4. Brain region-specific regulation of leptin expression and tissue-specific activation of STAT3 in rats exposed to DHT and/or flutamide for 1 week. A–D, Immunohistochemical staining for leptin was performed sections of (A) cerebral cortex, (B) hippocampus CA2, (C) paraventricular nucleus (PVN), and (D) the medial preoptic area (MPO) after 1 week of treatment with vehicle, DHT, flutamide, or DHT/flutamide. DHT exposure decreased the number of leptin-immunoreactive cells in the PVN and MPO. Images in (A1) are at higher magnifications than A. Data shown are representative of at least two independent experiments with similar results ($n=3$ rats/group). Scale bars, 100 μm. RSA, retrosplenial agranular; 3v, third ventricle. E and F, Western blot analysis of phospho-STAT 3 and α-tubulin expression in the hypothalamus (E) and mesenteric adipose tissue (F). Equal amount of proteins was separated on 4–12% one-dimensional Bis–Tris gels and immunoblotted with antibodies against phospho-STAT3 or α-tubulin. Band intensities were quantified by densitometry. Data are expressed as arbitrary densitometric units (ADU). Values are mean ± SEM ($n=6$ rats/group). $^{**}p<0.01$ vs. vehicle.

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