Androgen-Sensitive Changes in Regulation of Restraint-Induced Adrenocorticotropic Secretion between Early and Late Puberty in Male Rats

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Regulation of ACTH secretion changes between early (40 d) and late (60 d) puberty in male rats. We tested whether this occurs because of activating effects of testosterone on the brain. We measured testosterone and ACTH responses to repeated restraint in adrenalectomized, corticosterone-replaced rats entering and leaving puberty with or without treatment with flutamide, a nonsteroidal androgen-receptor antagonist. Flutamide increased testosterone. ACTH responses were high and suppressed by flutamide at 40 d. At 60 d, ACTH responses were low and increased by flutamide. On d 4, basal arginine vasopressin (AVP) mRNA was increased by restraint, but not age, in the medial parvocellular paraventricular nucleus (mpPVN) and medial amygdala and increased with age in the bed nucleus of the stria terminalis. We counted numbers of AVP-immunoreactive (AVP-ir) and corticotropin-releasing factor (CRF)-ir neurons. In medial amygdala, there was no change in AVP+ cells. With restraint, CRF+ cells in the central nucleus decreased at 40 d and increased at 60 d. Flutamide did not affect the response at 40 d but blocked restraint-induced increases at 60 d. After restraint, the bed nucleus of the stria terminalis AVP-ir correlated negatively with mpPVN CRF-ir at 40 d and with mpPVN AVP-ir at 60 d. In PVN, there were no effects on CRF+ cells. However, AVP+ cells increased only with restraint plus flutamide at 40 d and tended to increase with restraint and decrease with restraint plus flutamide at 60 d. We conclude that during puberty testosteronereinduces marked changes in regulation of neuropeptides in pathways known to determine autonomic, neuroendocrine, and behavioral responses to chronic stress. (Endocrinology 145: 59–70, 2004)

Pubertal development or adolescence in males encompasses a period of marked neural (1–3) and somatic growth and differentiation that renders pubertal individuals vulnerable to challenges (1–9). Accompanying and probably responsible for most of these pubertal changes is the increase in activity of the hypothalamo-pituitary-gonadal (HPG) axis, resulting in increased gonadal testosterone secretion.

We have recently shown remarkable differences in ACTH, testosterone, and metabolic responsiveness to chronic or repeated stressors in male rats at the beginning (40–45 d) and the end (59–65 d) of puberty (10–12). For example, exposure to repeated restraint causes rats entering puberty to defend body weight gain whereas those leaving puberty lose weight; ACTH responses to restraint are consistently greater in the younger rats. These responses of male rats entering and leaving puberty may evolve in a gender-specific manner. Because the rats entering puberty are not yet sufficiently grown to reproduce, the achievement of physical fitness and growth is promoted, whereas the already reproductively competent rats (60 d) are likely to allocate energy mainly in those strategies that promote reproductive success. However, to defend these goals in the face of adversity, other coping strategies would be expected to change as well (13).

Reciprocally inhibitory interactions occur at several levels between the hypothalamic-pituitary-adrenal (HPA) and HPG axes (14, 15; see refs.). Strong evidence relates the inhibitory effect of the HPG axis on the HPA axis function to the effects of testosterone on extrahypothalamic, testosterone-sensitive vasopressinergic pathways. Testosterone stimulates vasopressin (AVP) mRNA and peptide in the medial parvocellular paraventricular nucleus (mpPVN), the bed nucleus of the stria terminalis (BNST), and the medial amygdala (MeA) (16–27). The increases in extrahypothalamic vasopressinergic neurons appear to inhibit stress-induced secretion of ACTH-stimulating neuropeptides from the PVN. After restraint stress, testosterone inhibits both the numbers of c-Fos-immunoreactive (c-Fos-ir) cells in the mpPVN and ACTH secretion (24). Testosterone levels are strongly correlated with AVP content in the median eminence of stressed rats, suggesting inhibition of secretion (22, 28–30). However, corticotropin-releasing factor (CRF)- and AVP-expressing parvocellular neurosecretory neurons in the mpPVN do not contain androgen or estrogen receptors (31–34). Thus, it is likely that the inhibitory effects of testosterone on HPA axis responses to stress are indirect and mediated by its action on central neurons that are androgen and/or estrogen sensitive and stress responsive, with inputs to the mpPVN. In the mpPVN, AVP-expressing parvocellular neurons appear to be more sensitive to androgen influences than CRF neurons.

There is a known inhibitory input from the amygdala and
BNST to neuroendocrine cells in the PVN through γ-aminobutyric acid (GABA) (35, 36). GABA-containing CRF neurons in both sites change upon adrenalectomy or are required for components of ethanol withdrawal (37, 38). AVP-ir neurons in the amygdala and BNST are very responsive to gonadal hormones. It also seems likely that AVP-ergic cells in the amygdala and BNST coexpress GABA, and these cells may exert similar inhibitory functions on neuroendocrine cells in the mpPVN.

We previously proposed (see Ref. 12) that 1) stress-induced testosterone would increase AVP in the PVN and extended amygdala in adult but not pubertal male rats, 2) CRF in the PVN would be less responsive in 60-d- than in 40-d-old rats to the inhibitory effect of testosterone, 3) restraint-induced inhibition of testosterone in 40-d-old rats would result in a greater modulation of the HPA axis response by CRF than by AVP, and 4) blocking the effect of testosterone would increase ACTH responsiveness in 60-d-old but not in 40-d-old rats and would alter brain AVP.

To test these hypotheses we compared the effects of flutamide, a nonsteroidal androgen receptor (AR) antagonist, on responses to repeated restraint in rats entering (40 d) and leaving (60 d) puberty. Flutamide was chosen because nonsteroid antiandrogens interact only with the AR and therefore do not affect glucocorticoid receptors. All rats were adrenalectomized and replaced with exogenous corticosterone (ADX+B) to hold circulating corticosterone roughly constant to clarify the effects of manipulating testosterone activity.

Materials and Methods

Animals

Male Sprague Dawley rats delivered from Bantin and Kingman (GiloBay, CA) were used. Rats were individually housed in hanging wire cages under standard conditions of light (lights on at 0600 h and off at 1800 h) and temperature (22 ± 2 C) and left undisturbed for a minimum of 3 d to allow adaptation to the new environment. Animals were offered Purina Rodent Chow (diet 5008) and tap water (0.5% NaCl solution after Purina Rodent Chow). Rats were pair-housed at 6/group, 40–41 d old, 195 g body weight) and 61 d old, 290 g body weight) were used. All manipulations were performed between 0800 and 1300 h to minimize any circadian influence.

Surgery and hormone replacement (common to experiments 1 and 2)

Surgical procedures were performed in the morning under a rodent anesthetic cocktail of ketamine (Abbott laboratories, North Chicago, IL), xylazine, and acepromazine (The Butler Co., Columbus, OH) (77:1:5.15 mg/ml, respectively, 1 ml/kg ip). On experimental d 1, all rats were bilaterally ADX by the dorsal approach and replaced with one 100-mg corticosterone/cholesterol pellet (Sigma Chemical Co., St. Louis, MO, and Steraloids, Inc., Wilton, NH, respectively). The 40-d-old and 60-d-old rats were replaced with 25% corticosterone/75% cholesterol pellets and with 35% corticosterone/65% cholesterol (wt/wt), respectively. The difference in the amount of corticosterone replacement between 40-d- and 60-d-old rats was based on their different initial body weights and was designed to mimic mean plasma corticosterone concentrations achieved over a diurnal cycle in adrenal-intact rats (39). Ketoprofen (10 mg/kg, 0.2 ml/100 g, sc; Sigma) was injected at surgery to reduce postsurgical pain and improve recovery.

The results from rats in experiment 1 have been reported previously (12), with the exception of AVP mRNA content in brain. Because we were unable to detect AVP-ir cells by immunocytochemical detection in the MeA in experiment 2, we now report AVP mRNA content in the mpPVN, MeA, and BNST from these rats, for comparison with the immunocytochemical results in experiment 2.

Flutamide treatment in experiment 2

After adrenal surgery on d 1, before returning the rats to their home cages, 12 rats of each age group were injected with vehicle (20% dimethylsulfoxide/80% corn oil, vol/vol, 0.05 ml/100 g sc), and the other 12 rats were injected with flutamide [20 mg/kg, 0.05 ml/100 g sc (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide, Sigma)]. Vehicle or flutamide injections were repeated on d 2, 3, and 4 at 0800 h, 2 h before starting the stress protocol.

Repeated restraint (common to experiments 1 and 2)

On d 2–4, half of the rats in each age and treatment group were exposed to 3 h of restraint stress (1000–1300 h) (12). Immediately before restraint, and within 2 min of the time they were taken from the home cage, a blood sample (prestress, time 0) was collected from a scalp nick made over a lateral tail vein. These samples were considered to reflect basal conditions. Rats were immediately placed in restraint tubes, and blood samples were collected 30, 90, and 180 min later by gently dislodging the clot that had formed over the initial cut. Samples of 300 μl were collected into EDTA-coated capillary tubes (Sarstedt, Nümbrecht, Germany), kept on ice, and centrifuged at 4 C. Aliquots of plasma were stored at −20 C until hormone concentrations were assayed. After the 180-min sample was collected in restraint, the rats were returned to their home cages and left undisturbed until the next day. Control rats were not restrained, handled during the restraint period, and they had free access to food and saline. All rats were decapitated under basal conditions on the morning of d 5, 24 h after the last vehicle or flutamide injection. In experiment 1, brains (three to four per group) from four groups were analyzed: control and restrained rats at 40 and 60 d of age. Experiment 2 resulted in eight experimental groups: vehicle-control, vehicle-stress, flutamide-control, and flutamide-stress at 40 and 60 d of age (n = 6 rats/group). Body weight was recorded daily in the morning immediately before injections. Food intake was measured daily at 1100 h.

Tissue collection

All rats were killed on the morning of d 5 by decapitation within 10 sec after they had been taken from their cages. Trunk blood was collected in plastic EDTA-containing tubes and centrifuged in cold, and plasma was stored in aliquots at −20 C until determination of basal hormone concentrations. Mesenteric, sc, epididymal, and perirenal white adipose tissues as well as thymuses, kidneys, testes, and seminal vesicles were dissected, cleaned, and weighed. In experiment 1, brains were immediately frozen in ethanol cooled with dry ice and saved at −80 C until in situ hybridization assays. In experiment 2, brains were fixed by immersion in a 4% paraformaldehyde solution at 4 C for 2 wk and cryoprotected in a solution of 4% paraformaldehyde, 30% sucrose at 4 C for 2 d.

Plasma hormone assays

All plasma hormone concentrations were measured by RIA. Plasma corticosterone, testosterone, and estradiol kits were from ICN Biomedicals (Costa Mesa, CA). The insulin kit was from Linco (St. Charles, MO). The IGF-I kit was from Diagnostic Systems Laboratories (Webster, TX). The ACTH RIA used a specific antisera generously donated by Dr. William Engeland (University of Minnesota, Minneapolis, MN) at a final dilution of 1:120,000, and [125I]ACTH (Incstar, Stillwater, MN) was used as tracer.

Hybridization histochemical localization of AVP mRNA was performed using a 32P-labeled antisense cRNA probe transcribed from a full-length (1.2-kb) cDNA from a 230-bp cDNA fragment encoding the

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vasopressin-specific 3′ end (exon C) of AVP (kindly provided by Dr. D. Richter, University of Hamburg, Hamburg, Germany). The probe was labeled in a reaction mixture consisting of 1 μg of linearized plasmid, 5× transcription buffer (Promega, Madison, WI), 5 μCi of [32P]UTP, and 10 mm of each ATP, CTP, and GTP, 100 mm dithiothreitol, 40 U RNase inhibitor, and 30 U polymerase. The reaction was incubated for 2 h at 37°C and treated with DNase I for 15 min at 37°C. Thereafter, the probe was separated from unincorporated nucleotides over a Micro Bio-Spin column (Bio-Rad, Hercules, CA). The probe was diluted in hybridization buffer, and 100 μl of diluted probe was applied to each slide to yield approximately 2×106 cpm of labeled AVP probe per slide. Slides were then coverslipped and incubated overnight at 55°C. The next day, slides were dipped in 2× SSC to remove the coverslips. Thereafter, slides were washed with RNase A at 37°C for 30 min followed by a wash in RNase A buffer (consisting of 1 M Tris, 5 M NaCl, and 0.5 M EDTA) without any RNase A for an additional 30 min at 37°C. Next, slides were washed in 1× saline sodium citrate at room temperature (RT) followed by a wash in 0.5× standard saline citrate for 30 min at 60°C. Finally, slides were dehydrated in 70, 95, and 100% ethanol for 2 min each and air dried. Slides were then exposed to Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) for the appropriate time to detect signal in the PVN, BNST, and MeA. Subsequently, slides were coated with Kodak NTB2 liquid autoradiographic emulsion (diluted 1:1, Eastman Kodak, Rochester, NY) and exposed at 4°C in the dark with desiccant, the duration determined by the strength of signal on the x-ray film. Emulsion-dipped slides were developed with Kodak D-19 for 5 min, briefly rinsed in distilled water for 15 sec, fixed in Kodak fixer for 5 min, and then washed in running water for 45 min, all at RT. Slides were subsequently counterstained with cresyl violet for anatomical reference purposes, dehydrated in graded series of alcohol, placed in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). All control and experimental sections within the same brain region were hybridized at the same time. Semiquantitative densitometric analyses of the relative level of AVP mRNA in the PVN, the BNST, and the MeA were performed using a Macintosh-driven NIH Image software (Rasband, version 1.61). Grain counts over cells were also determined. This was assisted by landmarks made apparent with the cresyl violet counterstaining. Optical density measurements in individual rats were determined from averaged readings of sections showing the highest AVP signal and corrected for background. The number of slices analyzed for each rat varied from three to five depending on the region and distribution of the transcript.

**Immunocytochemistry**

Coronal sections of the forebrain were cut at 30 μm using a Leica SM2000R Microtome. All immunocytochemistry was done on free-floating sections and not on frozen sections or paraffin. Sections were collected in 0.05 μM Tris-buffered saline (TBS; 0.05 μM Trizma base, 0.9% NaCl, pH 7.6) solution. Sections were then treated with the following solutions: TBS buffer containing 10% methanol and 3% H2O2 for 10 min to remove blood, RT; three 20-min washes in TBS at RT; and rabbit polyclonal specific antiserum diluted in 2% gelatin and 0.5% Triton X-100 in TBS over a 1-h period. For each series of one in five sections, the antibodies used were anti-AVP, generously provided by Dr. R. Buijs (Netherlands Institute of Brain Research, Amsterdam, The Netherlands) and anti-CRF (70), generously provided by Dr. W. Vale (Peptide Biology Laboratories, Salk Institute, San Diego, CA). AVP and CRF antisera were used at a 1:4000 dilution. After incubation with the primary antisera, there were three 20-min washes in TBS at RT, addition of biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:400 in 2% gelatin and 0.5% Triton X-100 in TBS for 60 min at RT, and rabbit polyclonal specific antiserum diluted in 2% gelatin and 0.5% Triton X-100 in TBS over a 1-h period. After several washes in TBS at RT. Avidin-biotin peroxidase complex (Vector Elite Series, Vector) in 2% gelatin, 0.5% Triton X-100 in TBS was added for 60 min at RT and was followed by 3× 20 min washes in TBS at RT. For the development of immunostaining the sections were incubated for approximately 7 min in substrate (0.025% 3,3′-diaminobenzidine tetrahydrochloride in TBS containing 0.05% H2O2). The reaction was then stopped in TBS buffer. After several washes in TBS, sections were mounted on slides, air dried, and coverslipped with Permount. Each series of sections from the eight groups of rats (three brains/group) was run in a single set so that comparisons could be made across all groups. AVP mRNA expression was determined in the PVN, BNST, and MeA in rats from experiment 1. In experiment 2, AVP-ir cells were detected in the PVN and BNST; AVP-ir was also examined in the external layer of the median eminence, and CRF-ir was measured in the PVN and central nucleus of the amygdala (CeA).

To determine effects of age, restraint, and flutamide, the number of immunoreactive cell bodies was counted in every fifth section in a total of three animals per group. Immunoreactive cells were identified by hand counting them through the microscope by an observer who was blind to group assignment. When counting immunoreactive cells, at least three to four sections per animal were bilaterally analyzed. All identified AVP-ir or CRF-ir cells were counted, independently of the intensity of the staining observed, although rough records of the intensity of staining in the cells were recorded. In the PVN (bregma −1.80 to −1.88), CRF-ir and AVP-ir were performed in adjacent sections. AVP-ir neurons in the parvicellular PVN were differentiated from magnocellular AVP-ir neurons using both the anatomic control of cresyl violet adjacent stained sections and the distribution of CRF-ir in adjacent sections as a reference. Because results of CRF + cells in the CeA (bregma −2.3 to −2.8) were so remarkable, these cells were counted three times: twice independently by individuals through the microscope and once using computer-assisted identification of positive cells. The results of the three measures agreed within ±2 cells/group, and the direction and significance of the results were always similar. Final results were calculated as the mean of total cells per section.

**Statistics**

Three-way ANOVAs for repeated measures with age, treatment, and restraint as main factors and time as a repeated measure were used to analyze daily body weight, food intake, and hormone responses to restraint. Three-way ANOVA was also used to analyze mRNA and cell number in the studies of rat brains. When interactions were found to be significant (P < 0.05) or marginally significant (P < 0.1), additional comparisons were done using two-way ANOVAs (within age) followed by Student t tests (two-tailed) to assess statistical significance of treatment and/or restraint (Statview 5.0).

Linear regressions between AVP-ir in the BNST and both AVP-ir and CRF-ir in the PVN were determined on data from 40- and 60-d-old rats. Although we are aware that regressions need not explain direct causal relationships between variables, the regressions used were based on our hypothetical construct.

**Results**

**Efficacy and effects of flutamide treatment and restraint**

Table 1 shows results under basal conditions from the final day (d 5) of the experiment. Normalized seminal vesicle weight (an androgen target organ) was similar at the two ages, and flutamide treatment reduced seminal vesicle weight to a similar degree at both ages. Restraint in the 60-d-old rats increased seminal vesicle weight, possibly as a consequence of testosterone secretion during restraint (see below). Normalized testicular weight was greater in 40-d-old rats and was not affected by flutamide but was decreased by restraint. Plasma testosterone concentrations increased with age and with flutamide treatment. There was a marked effect of flutamide on plasma estradiol concentrations in the 40-d-old but not the 60-d-old rats; however, repeated restraint did not affect these concentrations.

There were no effects of treatment, age, or restraint on relative mesenteric white adipose tissue weight. Insulin and IGF-1 concentrations differed as a function of age and restraint, and there was a tendency for an interaction but no significant effect of flutamide treatment.

Normalized thyamus weight (a corticosteroid target organ) was heavier in the younger rats, but there was no effect of repeated restraint or of flutamide treatment. Plasma ACTH
and corticosterone concentrations were affected by age (P ≤ 0.0002), but there was no interaction between treatment and restraint (P = 0.154). The 40-d-old rats had significantly higher ACTH and lower corticosterone concentrations than 60-d-old rats (ranges, 3–6 μg/dl and 4–9 μg/dl, respectively). The concentration of corticosterone in the pellets used in 40-d- and 60-d-old rats differed, and this may partially explain the age effect on both ACTH and corticosterone. Additionally, the higher levels of ACTH at time 0 might be the consequence of greater responsiveness of ACTH in 40-d-old rats to the flutamide injection 2 h earlier. These replacement doses were chosen based upon age-dependent differences in body weight and previous experiments (10). In 40-d-old rats restraint decreased plasma corticosterone, independently of treatment, probably as a result of restraint-induced increased corticosterone clearance. In 60-d-old rats, however, stress increased plasma corticosterone only in flutamide-treated rats.

**Plasma testosterone responses to restraint**

The time course of plasma testosterone responses to restraint during the 3 d of restraint is shown in Fig. 1 (left panels; note the expanded scale for younger rats). Testosterone concentrations were initially low in the rats entering puberty at 40 d, and flutamide treatment both increased initial values and stimulated testosterone responses to restraint by d 3, but to a moderate extent (<5 ng/ml). By contrast, in rats leaving puberty at 60 d, initial testosterone concentrations were higher, both groups responded to restraint with testosterone secretion, and flutamide treatment markedly enhanced restraint-induced testosterone secretion (10–15 ng/ml). Thus, the older rats appear to have brains that are more sensitive to AR blockade under basal conditions and during restraint.

The cumulative area under the curve (AUC) for the responses to restraint at 30, 90, and 180 min (different from 0 time) was calculated for each day of restraint (Fig. 1, right panels). There were significant effects of age (P < 0.0001) and flutamide treatment (P = 0.0004) and an interaction between both main factors (P = 0.0014). The interaction between days and treatment was also significant (P = 0.05). On the last day, both 40- and 60-d-old rats treated with flutamide had larger testosterone responses to restraint than those treated with vehicle (P = 0.0121 and P = 0.0256, respectively). The 40-d-old rats treated with flutamide had a larger testosterone response to restraint on the third compared with the first day (P = 0.0235). On the second day of restraint, 60-d-old rats treated with flutamide had larger testosterone responses than those treated with vehicle (P = 0.0131).

**Plasma ACTH concentrations during restraint (Fig. 2)**

ACTH responses of high amplitude (~5 ng/ml) occurred on each day of restraint in the rats entering puberty (40 d), and flutamide treatment had little effect, although because of an increase in 0-time levels, it appeared to inhibit the responses (Fig. 2, top, left, and right panels). By contrast, the rats leaving puberty (60 d) had much smaller ACTH responses to restraint (~1–2 ng/ml), and flutamide increased the magnitude of the responses without affecting 0-time concentrations (Fig. 2, bottom, left, and right panels). Compared with our previous data (12), these differ in that the 40-d-old rats did not have increasingly diminished ACTH responses to restraint with repetition.

The cumulative AUC for the ACTH response to restraint at 30, 90, and 180 min was calculated for d 1, 2, and 3 (Fig. 2, right panels). There were significant effects of the interaction between age and treatment (P = 0.0097). There was a significant effect of days (P = 0.0119), an interaction between days and age (P < 0.0001), and an interaction among age, treatment, and days (P = 0.0055). On the third day, 40-d-old rats treated with flutamide had smaller ACTH responses to restraint than those treated with vehicle (P = 0.0317) and tended to have smaller ACTH responses to restraint on the second than the first day (P = 0.0564). The effect of flutamide was significant on the third day compared with the first day (P = 0.0189). The 60-d-old rats treated with flutamide had larger ACTH responses than those treated with vehicle on the second and third days of restraint (P = 0.046 and P = 0.0098, respectively). The 60-d-old rats treated with flutamide had larger ACTH responses on the third compared to the first day (P = 0.0235) and the second day (P = 0.0897).
with first and second days (P = 0.0284 and P = 0.0375, respectively). Blockade of ARs altered regulation of ACTH secretion during restraint in different ways that were age dependent. Flutamide may have inhibited the ACTH response in the rats entering puberty and clearly stimulated the ACTH response in rats leaving puberty.

**CRF-ir and AVP mRNA in the amygdala**

CRF cells in CeA are shown in Fig. 3A. Cells were counted through the microscope and were distinct from background (see high-magnification inset). There was a significant interaction between age and restraint (P = 0.0014) on CRF cell number in the CeA (Fig. 4A). Restraint decreased the numbers of CRF-ir-positive cells in 40-d-old rats, an effect that was independent of antiandrogen treatment (P = 0.021). However, in 60-d-old rats there were significant effects of treatment (P = 0.0105), restraint (P = 0.0073), and the interaction between treatment and restraint (P = 0.0152). Restraint increased CRF-ir cells in vehicle-treated but not in flutamide-treated rats (Fig. 4A).

The numbers of AVP mRNA-expressing cells in the MeA were counted as numbers of particles (Fig. 3D). There was a significant effect of age (P = 0.0334) with no significant effect of restraint or interaction on the numbers of AVP mRNA-expressing cells in the MeA from rats of experiment 1 (Fig. 4B). The numbers of grains per cell did not differ among groups (not shown). We were unable to detect AVP-ir cells in the MeA in experiment 2.

**AVP-ir in the BNST**

In experiment 1, there were significant effects of age on AVP mRNA in the BNST; 40-d-old rats had fewer visible cells than 60-d-old rats (Figs. 5, B and C, and 6A). The numbers of grains per cell did not differ as a function of the group (not shown). There were no effects of age on the number of AVP-ir cells in the BNST (Figs. 5A and 6B).

**CRF-ir, AVP mRNA, AVP-ir in mpPVN**

Cells were counted in the mpPVN, which is known to project to the median eminence and to be the neuroendocrine motor neurons for ACTH synthesis and secretion (40, 41). There was no significant effect of age, but restraint significantly increased PVN AVP mRNA content at both ages in experiment 1 (Fig. 7C). The numbers of grains per cell did not differ as a function of the group (not shown). In experiment 2, there were significant interactions between age and treatment (P = 0.0206) and among age, treatment, and restraint (P = 0.0121) in the numbers of AVP-ir cells in the PVN (Fig. 7B). In 40-d-old rats, flutamide markedly increased AVP-ir cell number (P = 0.006), and both restraint and the interaction between flutamide and restraint tended to be significant (P = 0.096 and P = 0.0715, respectively). Restraint in-
increased the number of AVP-ir cells in flutamide-treated (P = 0.044) but not in vehicle-treated rats at 40 d. In 60-d-old rats the interaction between treatment and restraint tended to be significant (P = 0.0594). The numbers of AVP-ir cells tended to increase in vehicle-treated and tended to decrease in flutamide-treated rats exposed...
to restraint (Fig. 7B). There were no significant effects of age, restraint, or flutamide treatment on the number of visible CRF-ir cells in the mpPVN (Fig. 7A).

AVP-ir in the median eminence

Restraint significantly decreased AVP-ir density in the internal layer of the median eminence that contains axons from magnocellular AVP-ergic neurons but had no significant effects on AVP-ir in the external layer that contains axons from parvicellular neurons. Although there was no significant effect at 40 d, in 60-d-old control rats, there was a significant positive relationship between testosterone and AVP-ir in the external zone of the median eminence as reported previously (15) (not shown).

Correlation between posterior BNST-AVP and mpdPVN CRF and AVP

The BNST has strong inputs to the neuroendocrine hypothalamus, which are probably inhibitory (42, 43). Therefore, we analyzed the correlation between the numbers of AVP-ir-positive cells in the BNST and the numbers of CRF-ir-positive and AVP-ir-positive cells in the neuroendocrine hypothalamus (Fig. 8). Under nonstressed conditions there were no significant correlations between AVP-ir cells in BNST and CRF-ir or AVP-ir in PVN in rats of either age ($P$ values range from 0.2–0.6). There was, however, a strong negative correlation in previously restrained 40-d-old rats between AVP-ir cell number in BNST and CRF-ir in mpdPVN ($r^2 = 0.85; P = 0.02$; Fig. 8, left), but not AVP-ir cell number ($P > 0.38$) in the mpPVN. By contrast, there was an equally strong negative correlation in 60-d-old rats that had been restrained between

FIG. 4. Age-dependent sensitivity of neuropeptide responses to both restraint stress and to androgen antagonist treatment in amygdala. A, Number of CRF-ir cells in the CeA of 40-d-old (left panel) and 60-d-old (right panel) rats that were ADX+B, treated with either vehicle or flutamide, and exposed to repeated restraint (see experiment 2). Results are shown as mean ± SEM ($n = 3$ rats/group). Repeated restraint induced a decrease in the number of CRF-ir cells in 40-d-old rats independently of vehicle or flutamide treatment. In 60-d-old rats, however, repeated restraint increased the number of CRF-ir cells in vehicle-treated rats (see also Fig. 3, A and B), an effect that was completely blocked by the AR antagonist. B, Number of AVP-expressing neurons in the MeA of 40-d-old (left panel) and 60-d-old (right panel) rats that were ADX+B and exposed to repeated restraint. Restraint increased the number of AVP mRNA-expressing neurons similarly at both ages. Results are shown as mean ± SEM ($n = 3$–4 rats/group). *, $P < 0.05$ compared with controls.

FIG. 5. A, Bright-field photomicrographs showing AVP-ir cell bodies and dendrites in the posterior BNST (bregma = –1.30 to –1.40). B and C, Bright-field photomicrographs showing AVP mRNA expression in the anterior (B) and posterior (C) BNST. Analysis of AVP mRNA expression in this cell group required individual cell- and grain-counting procedure. ac, Anterior commissure, f, fornix; 3v, third ventricle.
AVP-ir cell number in BNST and AVP-ir, \( r^2 = 0.86; P < 0.01 \); (Fig 8, right) but not CRF-ir \( (P = 0.29) \) cell number in the mpPVN.

**Discussion**

The results of these experiments confirm the marked differences in regulation of testosterone and ACTH secretion after repeated restraint stress between rats entering (40 d) and leaving (60 d) puberty (12). As hypothesized (12), treatment with the nonsteroidal AR antagonist flutamide altered the repeated ACTH responses to restraint in an age-dependent manner. Restraint stimulated testosterone in 60-d-old but inhibited it in 40-d-old rats. Flutamide treatment increased testosterone responses to repeated restraint at both ages. Restraint-induced ACTH was stimulated by flutamide in 60-d- but not 40-d-old rats that were ADX+B, treated with either vehicle or flutamide, and exposed to repeated restraint. Results are shown as mean ± SEM \( (n = 3 \) rats/group) \( *, P < 0.05 \).

The effects of age, flutamide, and restraint on sex hormone concentrations and metabolism

Based on the flutamide-induced inhibition of testosterone target tissue weight, the dose of antiandrogen was equipotent at the two ages. Plasma testosterone concentrations increased with age and flutamide, although the flutamide effect was greater in 60- than in 40-d-old rats. These results are in agreement with the known increase of testosterone that occurs during sexual development in the male rat (45–60 d) and with the decreased sensitivity of the HPG axis to testosterone negative feedback that occurs at puberty. Circulating androgens are converted to estrogens by the action of...
The cytochrome P450 enzyme, aromatase, found in many peripheral tissues and in brain (44, 45). Androgens regulate aromatase pretranslationally and AR activation is correlated with the induction of aromatase activity constituting a feed-forward system where testosterone is both the positive regulator and the major substrate of aromatase. Flutamide is known to increase plasma concentrations of estradiol, as it did here, reflecting both peripheral aromatization of testosterone and testicular estrogen production. An increase of plasma estradiol concentrations induced by flutamide, but unaffected by restraint, occurred in 40- but not in 60-d-old rats, congruent with the finding that aromatase activity and mRNA in peripheral reproductive tissues is higher during puberty than in adulthood (46). Moreover, these developmental changes in aromatase activity are probably related to the importance, particularly in male pubertal development, of not only androgens but also estrogens on bone formation, carbohydrate and lipid metabolism, as well as neural and behavioral maturation (47). Although age and restraint affected body weight gain as in our earlier experiments (12), the short duration of flutamide treatment did not affect this response. Age affected food intake as previously shown (12), but flutamide did not affect food intake.

Effects of age and flutamide on brain neuropeptide responses

In several sites, opposite effects were observed in 40- and 60-d-old rats on changes in neuropeptide expression after the stressor of repeated restraint. This clearly shows that during the 3-wk pubertal transition, from the juvenile to the reproductively competent state, marked changes in brain regulatory systems have taken place. These are probably related to the means of meeting new demands for survival and production of stress hormone responses to repeated restraint. This clearly shows that during the 3-wk pubertal transition, from the juvenile to the reproductively competent state, marked changes in brain regulatory systems have taken place. These are probably related to the means of meeting new demands for survival and production of stress hormone responses to repeated restraint.

Age increases AVP in the MeA and BNST

Age increased AVP mRNA cell number in the MeA and the AVP-ir cell number in the BNST, effects that were not altered by the AR antagonist. Therefore, our results support previous reports showing that in male rodents, AVP transcription and AVP-ir neurons in these nuclei are stimulated by testosterone (16-19) and increased in adulthood (53). These effects are mediated by estrogen after aromatization of testosterone to estradiol, and estrogen receptors are present in both BNST and medial amygdala. Moreover, knockout mice lacking a functional aromatase enzyme (ArKO) have a marked loss of AVP-ir in the BNST and MeA of colchicine-treated brains compared with the wild type (54), suggesting that many of our observed effects were mediated by the aromatase activity constituting a feed-forward system where testosterone is both the positive regulator and the major substrate of aromatase. Flutamide is known to increase plasma concentrations of estradiol, as it did here, reflecting both peripheral aromatization of testosterone and testicular estrogen production. An increase of plasma estradiol concentrations induced by flutamide, but unaffected by restraint, occurred in 40- but not in 60-d-old rats, congruent with the finding that aromatase activity and mRNA in peripheral reproductive tissues is higher during puberty than in adulthood (46). Moreover, these developmental changes in aromatase activity are probably related to the importance, particularly in male pubertal development, of not only androgens but also estrogens on bone formation, carbohydrate and lipid metabolism, as well as neural and behavioral maturation (47). Although age and restraint affected body weight gain as in our earlier experiments (12), the short duration of flutamide treatment did not affect this response. Age affected food intake as previously shown (12), but flutamide did not affect food intake.

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Local estrogen biosynthesis by aromatase occurs in neurons as well as in the periphery and is required for the full expression of male sexual behavior in rats. The progressive increase of gonadal hormones during pubertal development is associated with reduced AR and basal aromatase activity in the medial preoptic area but not in other regions such as the BNST and amygdala (48-50). However, whether peripheral and/or brain aromatase activities are affected by stress and whether that is age dependent is unknown. Thus, the age-dependent differences in the neuroendocrine responses to restraint reported here are probably related to age-dependent differences in both androgenic and estrogenic effects of testosterone in brain. It does seem clear from these results that new, testosterone- and/or estradiol-sensitive pathways mediating chronic stress responses in adulthood mature or become functional during the pubertal transition from the juvenile to the adult state.

At this point, our studies have just begun. We immunocytochemically analyzed AVP-ir and CRF-ir cell numbers on fixed brains from decapitated, non-colchicine-treated rats, and we were at the mercy of the small numbers of cells that we could see; we probably did not detect the total cell number in any site. However, because the same structures in all brains were immunostained simultaneously, it is likely that comparison among groups is justified. Moreover, it is quite possible that the ratio between AVP and CRF expression may have changed between the two ages, making visualization of one or the other peptide more likely. We were not able to visualize AVP-ir cells in the MeA, and we did not stain CRF-ir cells in the BNST. The cells in both sites are likely to be important in regulation of chronic stress responses (see Refs. 14 and 26). Because we did not double-stain for CRF and AVP, we cannot say that the AVP-ir and CRF-ir were in the same cells in the mpPVN; however, it is likely that there was at least some AVP-ir in CRF-ir cells (51, 52). Despite these caveats, the results prompt our conclusion that increasing testosterone secretion with age defines the numbers of neuropeptide-expressing cells in the limbic-hypothalamic circuit important for central control of neuroendocrine responses to repeated restraint.
Age-dependent effects of restraint on AVP in the MeA

Repeated restraint increased AVP mRNA-positive cell numbers in the MeA. AVP in the MeA has been implicated in a variety of social and sexual behaviors as well as aggression and is regulated by circulating gonadal steroids (55). However, inspection of the figures shows that 60- but not 40-d-old rats tended to have a greater increase in cell number in MeA, after restraint-induced increases in testosterone secretion. We do not know what effect flutamide treatment might have had in the MeA, because only stress and age could be compared, using AVP mRNA expression. However, studies in male hamsters have shown that chronic treatment with anabolic androgen steroids (AASs) during pubertal development facilitates offensive aggression by affecting vasopressinergic function (56).

Interactions among age, flutamide, and restraint

There was a definite restraint-flutamide interaction on peptide-positive cell numbers between rats of 40 and 60 d of age. These results suggest that major reorganization of stress-sensitive pathways occur during puberty, as has been shown in other behavioral, neurochemical, and anatomic studies (1–9, 57–60). The prefrontal cortex, amygdala, and nucleus accumbens are structures in which innervation continues to change throughout puberty (2, 3, 61–63). It seems likely that this changing pattern of innervation is both gender and hormone specific and relevant to the regulation of responses to challenge.

CRF in the CeA

CRF in the CeA is part of a circuitry that mediates neuroendocrine, autonomic, and behavioral responses to stress. Although ARs and estrogen receptors are found in the CeA, they are not colocalized in CRF neurons but are found in adjacent cells; CRF in the CeA can be indirectly modulated by sex steroids (64). In contrast, CRF-containing neurons of the CeA can be directly modulated by corticosterone through the glucocorticoid receptors that they contain (65). In the presence of an intact adrenocortical system, basal expression of CRF mRNA levels in the CeA is negatively related to plasma testosterone concentrations, but testosterone does not alter the ADX-induced reduction in CRF mRNA expression in the CeA in adrenalectomized rats (26). In the CeA, there were no significant age-related effects on basal CRF-ir. However, in the 40-d-old rats, restraint decreased CRF-ir cells independently of flutamide whereas in the 60-d-old rats, restraint increased CRF-ir cells, and the effect was blocked by flutamide. Thus, the results suggest that in gonadal intact rats, stress-induced CRF in the CeA may be partially independent of the adrenocortical response to stress and positively regulated by testosterone. Moreover, the age differences in restraint-induced testosterone responses may be responsible for the stress-induced differences in CRF-ir cell number between the ages. Chronic treatment with the AAS nandrolone decanoate has been shown to increase behavioral responses to stress and the density of c-Fos-ir neurons in the CeA of adult male guinea pigs (66), suggesting that AAS might enhance the activity of the CeA. Thus, regulation of CRF in the CeA by testosterone appears to be either inhibitory or stimulatory depending on whether the CeA is sampled under basal or stress conditions. Whether these effects are mediated in an age-dependent way through either the androgenic and/or estrogenic effects of testosterone, some sex-steroid-sensitive behaviors have been shown to be specifically androgen sensitive (21, 67–70), and some behaviors have been shown to be specifically testosterone dependent in the amygdala and BNST (71–73).

AVP in the mpPVN

In the mpPVN, there were, again, opposite effects of flutamide treatment on responses to restraint at 40 and 60 d of age. In vehicle-treated rats, restraint did not change visible AVP+/cell numbers in 40-d-old rats, but similarly to the pattern in CRF+/cells in the CeA, repeated restraint increased AVP+/cells in 60-d-old rats. Flutamide treatment allowed increased AVP+/cell number in the restrained 40-d-old rats but blocked the changes after restraint that occurred in the 60-d-old rats, resembling the pattern observed in CRF+/cells in the CeA.

BNST-PVN and regulation of the HPA axis responses to restraint

We correlated BNST AVP-ir with mpPVN AVP-ir and with mpPVN CRF-ir in 40- and 60-d-old rats. We found strong correlations that support our hypotheses. Thus, AVP-ir in the BNST was negatively correlated with mpPVN CRF+/cells in 40-d-old and with mpPVN AVP+/cells in 60-d-old rats. We assume that many of the cells visualized in mpPVN using AVP-ir also contain CRF, and we suggest that there is a marked inhibitory input to the mpPVN from vasopressinergic cells in the BNST. As rats progress from early to late puberty, they may shift their hypothalamic regulation of chronic ACTH responses from a CRF-dominated to an AVP-dominated response through maturation of testosterone-sensitive AVP-ergic pathways.

Because AVP within the PVN inhibits CRF and ACTH secretion (74), restraint-induced increases in both testosterone and, indirectly (because there are no ARs in the mpPVN), AVP+/cells in PVN may explain the typical reduction in the magnitude of ACTH responses that occur in 60-d-old and older male rats. Of course, it is no surprise that the limbic network known to be important in regulating HPA responses is similar or identical with the limbic pathway that is androgen sensitive and regulates sex behavior (15). This would be expected because of the many different autonomic, neuroendocrine, and behavioral responses that are common and change between early puberty and adulthood.

In summary, we have shown that age-dependent testosterone responses to repeated bouts of restraint differentially affect neuropeptide expression in limbic and hypothalamic cell groups involved in regulation of the HPA axis and ACTH secretion in response to restraint. Because of the differential responses that occurred in the amygdala, we anticipate that there will also be marked behavioral and autonomic effects.
that are mediated by stressors that induce testosterone secretion in adult but not juvenile rats (14). We expect that the overall effects of persistent, adversity-induced increases in testosterone secretion in 60-d-old rats will be found to shape more male-typical behaviors such that reproductive capacity is defended, possibly at the cost of other physiological systems. Restraint-induced decreases in testosterone in 40-d-old rats may be considered as an adaptive response associated with the maintenance of a positive energy balance in response to challenge (11). Moreover, we suspect that abnormally increased testosterone responses to stress during adolescence may result in the development of psychopathologies such as aggressive behavior or depression. Finally, as a more technical consideration, it is important to note that the increase in body weight from 40–60 d of age in male rats is approximately 80 g. However, rats weighing 200 g (40 d) have very different neuroendocrine and behavioral responses to both cold (11) and restraint (12) than rats weighing 280 g (60 d). Thus, those who study male rats should use caution in selecting the weight range to be employed.

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