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Gender and Puberty Interact on the Stress-Induced Activation of Parvocellular Neurosecretory Neurons and Corticotropin-Releasing Hormone Messenger Ribonucleic Acid Expression in the Rat

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Individual variations in hypothalamic-pituitary-adrenal (HPA) function are most evident at or beyond the time of puberty, when marked changes in sex steroid release occur. To explore the nature by which gender differences in HPA function emerge we examined in prepubertal (~30-d-old) and postpubertal (~60-d-old) male and female rats HPA activity under basal conditions and in response to 30 min of restraint. Within the ACTH-regulating, medial parvocellular portion of the paraventricular nucleus, restraint-induced Fos protein and arginine vasopressin heteronuclear RNA were lower in 60- than in 30-d-old males. No such age-related shift in the response of these synaptic and transcriptional markers of cellular activation occurred in female rats. Basal CRH mRNA expression levels in the paraventricular nucleus increased with age in female but not male rats. Conversely, only male rats showed an age-related increase in basal CRH mRNA in the central amygdala, suggesting that neuronal and neurosecretory CRH-expressing cell types are subject to different pubertal and gender influences. We conclude that gonadal regulation of the HPA axis develops via distinct mechanisms in males and females. Puberty-related shifts in parvocellular neurosecretory function in males are emphasized by stress-induced shifts in neuronal activation, whereas biosynthetic alterations dominate in female rats. (*Endocrinology* 146: 137–146, 2005)

HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) activity varies as a function of gender, age, reproduction, social status, and disease (1–3). All of these influences are linked if not dictated by sex steroids, including testosterone and estrogen. In adult mammals, including humans and rodents, the HPA axis responds to variations in circulating sex steroid concentrations. Estrogen exerts, for the most part, stimulatory effects on stress-induced ACTH and glucocorticoid release, whereas testosterone acts to inhibit stress HPA activity (4, 5).

In addition to the established effects of sex steroids on HPA function exerted during perinatal development and adulthood (6, 7), a few but seminal number of studies now make evident the dynamic nature by which the gonadal and adrenal axes interact during puberty (8–10). Furthermore, this transition period is not only marked by large changes in the basal release of adrenal and gonadal steroids but also by major shifts in stress-induced HPA activity. For example, relative to adults (≥60 d old), prepubertal male rats (≤30 d old) show higher if not longer plasma ACTH and corticosterone responses to ether, intermittent foot shock, and acute 30-min restraint exposure (11–13). This pubertal shift in HPA responsiveness is not only coincident with elevations in plasma testosterone levels but also with an emergent sensitivity to testosterone (14). Thus, it appears that the inhibitory effects of testosterone on HPA function in males become operational at the time of puberty.

Our previous findings have established various criteria with respect to studying how androgens act to inhibit stress-induced HPA activity centrally within the medial parvocellular, ACTH-regulating arm of the paraventricular nucleus (PVN). In the male rat, stress-induced elevations in Fos protein and arginine vasopressin (AVP) heteronuclear (hn) RNA transcriptional activation in adult male rats are elevated by castration (15), effects readily reversible with testosterone replacement in a dose-dependent manner (5, 16). Critically, these indices of synaptic and transcriptional activation also vary strongly and negatively with plasma testosterone levels in gonadal-intact rats. Taken together, these findings suggest that individual differences in HPA output are explained by normal variations in plasma testosterone registered by stress-related inputs to the PVN.

In the present study, we examined the extent to which puberty-related changes in HPA function are likewise paralleled by shifts in the stress-induced activation of medial parvocellular neurons. This was achieved by contrasting the stress-induced patterns of cellular activation displayed by prepubertal and postpupertal male rats exposed to 30 min of restraint. If an inhibitory testicular influence becomes operational at puberty, then this should be reflected by a relative age-related decrease in medial parvocellular recruitment. Because maturational shifts in HPA output could also be produced by changes in the drive to synthesize CRH, relative levels of CRH mRNA in the PVN were also assessed. This analysis was extended to the central nucleus of the amygdala (CeA), a key integrator of autonomic, behavioral, and neu-

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Abbreviations: AVP, Arginine vasopressin; CeA, central nucleus of the amygdala; hn, heteronuclear; HPA, hypothalamic-pituitary-adrenal; ir, immunoreactivity; PVN, paraventricular nucleus; SSC, standard saline citrate.

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roendocrine responses to stress (reviewed in Refs. 17–19), to differentiate potential pubertal influences on neurosecretory *vs.* nonneurosecretory, CRH-expressing cell types. Finally, because females emerge from puberty showing marked elevations in adrenal output, tissue from 30- and 60-d-old female rats were similarly employed to assess possible sex differences and ovarian influences on puberty-related shifts in medial parvocellular activity.

Materials and Methods

Animals

The animals used were male and female Sprague Dawley rats (Charles River Canada, St. Constant, Quebec, Canada). Male and female rats were sampled over two nested periods, from 28–32 and 58–62 d of age, representing prepubertal and postpubertal age groups, respectively. These sampling periods are in step with previous developmental landmark studies in the Sprague Dawley rat (Charles River Laboratories: http://www.criver.com/techdocs/index.html) and functional neuroendocrine studies (20–23) and as verified here by RIA, consistent with major concentration shifts in basal plasma corticosterone, testosterone, estrogen, progesterone, and LH levels (see data and Table 1). Rats were housed with the same sex, two per cage, under controlled temperature and lighting conditions (12-h light, 12-h dark cycle; lights on at 0600 h), with food and water available *ad libitum*. Testing in all cases was performed during the light phase of the cycle, beginning at 0900 h and ending at 1000 h. All protocols were approved by the University of British Columbia Animal Care Committee.

Rats were weighed and handled daily (presented to, but not placed within, restrainers) for 6–10 d before stress testing to monitor their health and to habituate the animals to the restraint procedure. On test days, the animals were anesthetized with a lethal dose of chloral hydrate (35% wt/vol; 1ml per 100 g body weight; ip injection) and perfused either immediately after removal from the home cage (basal group) or at the end of a single 30-min exposure to restraint (stress group). Mean final body weights in 30-d-old male and female rats were 101.9 ± 3.8 and 93.9 ± 3.3 g, respectively; 60-d-old male and female rats were 326.8 ± 4.4 and 202.2 ± 2.7 g, respectively. Given this expected body weight and size difference between 30- and 60-d-old rats, the degree of restraint was controlled by employing Plexiglas restrainers of two different sizes: 5.0 × 12.5 cm and 6.3 × 15 cm (Kent Scientific, Litchfield, CT).

As verified by corneal, pedal, and tail-pinch reflexes, deep anesthesia was reliably achieved within 45–60 sec of chloral hydrate administration. Blood samples were obtained from the right atrium, just before perfusate delivery via the ascending aorta, and collected into ice-chilled EDTA-treated tubes, centrifuged at 3000 × *g* for 20 min, and finally stored at –20 C until assayed for plasma hormones. Saline (0.9%) and fixative (4% paraformaldehyde) were delivered over 5 and 20 min, respectively, at a flow rate of 20–25 ml/min. Brains were then postfixed for 5 h and cryoprotected overnight with 10% sucrose in 0.1 M potassium PBS (KPBS) (pH 7.3) before slicing. Five 1-in-5 series of frozen coronal sections (30 μm) throughout the length of the hypothalamus were collected and stored in antifreeze/cryoprotectant (30% ethylene glycol and 20% glycerol in 0.05 M sterile KPBS) at –20 C until processing.

TABLE 1. Puberty-related shifts in plasma indicators of basal gonadal and adrenal function

	Prepubertal (~30 d old)	Postpubertal (~60 d old)
Male		
Testosterone	0.05 ± 0.13 ng/ml	2.30 ± 0.66 ng/ml[a]
Progesterone	14.90 ± 6.20 ng/ml	15.90 ± 6.50 ng/ml
LH	0.22 ± 0.02 ng/ml	0.83 ± 0.20 ng/ml[a]
Corticosterone	3.58 ± 1.70 μg/dl	3.85 ± 1.99 μg/dl
Female		
Estrogen	44.72 ± 3.80 pg/ml	93.10 ± 19.6 pg/ml[b]
Progesterone	1.50 ± 0.58 ng/ml	47.90 ± 6.39 ng/ml[a]
LH	2.20 ± 0.39 ng/ml	1.40 ± 0.37 ng/ml
Corticosterone	1.85 ± 0.43 μg/dl	15.70 ± 3.35 μg/dl[a]

[a] $P < 0.01$; [b] $P < 0.05$ *vs.* prepubertal (n = 4 rats per basal group).

Hormone measurements

Plasma testosterone (25–75 μl sample volumes), estrogen (25 μl), progesterone (12.5 μl), LH (50 μl), corticosterone (5 μl plasma diluted 1:200), and ACTH (50 μl) concentrations were measured using commercial RIA kits (ICN Biomedicals, Costa Mesa, CA) with slight procedural modifications. These included tripling the sample volumes for testosterone measurements in prepubertal male rats, using half the suggested sample volumes for plasma progesterone, and employing additional standard points for the estrogen RIA standard curve. The intra- and interassay coefficients of variation for all of the assays typically ranged from 3–6 and 8–12%, respectively. ^{125}I-labeled ligands were used as tracer in all cases. The testosterone antibody cross-reacts 100% with testosterone and slightly with 5α-dihydrotestosterone (3.40%), 5α-androstane-3β, 17β-diol (2.2%), and 11-oxotestosterone (2%). The standard curve ED_{50} for the testosterone RIA was 1.0 ng/ml, with a detection limit of 0.1 ng/ml. The estrogen antibody cross-reacts 100% with 17β-estradiol and slightly with estrone (20%), estriol (1.5%), and 17α-estradiol (0.7%). The standard curve ED_{50} for the estrogen RIA was 75 pg/ml, with a detection limit of 5 pg/ml. The progesterone antibody cross-reacts 100% with progesterone and slightly with 20α-dihydroprogesterone (5.4%) and 17α-hydroxyprogesterone (0.7%). The standard curve ED_{50} for the progesterone RIA was 20 ng/ml, with a detection limit of 0.5 ng/ml. The LH antibody cross-reacts 100% with LH and slightly with FSH (8%), GH (5%), and prolactin (2.5%). The standard curve ED_{50} for the LH RIA was 2.7 ng/ml, with a detection limit of 0.5 ng/ml. The corticosterone antibody cross-reacts 100% with corticosterone and slightly with desoxycorticosterone (0.34%) and testosterone and cortisol (0.10%) but does not react with the progestins or estrogens (<0.01%). The standard curve ED_{50} for the corticosterone RIA was 17 μg/dl, with a detection limit of 1.25 μg/dl. The ACTH antibody cross-reacts 100% with $ACTH_{1-39}$ and $ACTH_{1-24}$, but not with β-endorphin, α- and β-MSH, and α- and β-lipotropin (all <0.8%). The standard curve ED_{50} for the ACTH RIA was 85 pg/ml, with a detection limit of 25 pg/ml.

Characterization of restraint-responsive neurons

Fos immunohistochemistry. Restraint-responsive neurons in the PVN were localized using Fos immunoreactivity (Fos-ir) as a marker of synaptic activation. Fos-ir was detected using a conventional avidin-biotin-immunoperoxidase procedure (Vectastain Elite ABC kit; Vector Laboratories, Burlington, CA) to localize a primary antiserum (1:23,000) raised against amino acids 4–17 of human Fos protein (Oncogene Research Products, Boston, MA). Specific staining was abolished by absorbing the primary antiserum with 50 μM synthetic Fos (Oncogene Research Products). Light-level images were captured using a Hamamatsu optical system coupled to a Macintosh computer running Open Lab imaging and measuring software (Quorum Technologies, Guelph, Ontario, Canada). Fos-ir cell counts were taken by an observer blind to animal status in regularly spaced (150-μm) intervals through the rostrocaudal extent of the paraventricular cell group and corrected for double-counting error. Positive cells were identified as those expressing a black nuclear reaction product. Discrete localization of Fos-ir profiles to the medial parvocellular (neuroendocrine anterior pituitary-regulating) population of the PVN was assisted by redirected sampling of an adjacent series of thionin-stained sections (24). Cell number estimates were generated by counting bilaterally the number of Fos-positive cells through the medial parvocellular cell population, averaged by dividing cell counts by slice number, and corrected for double-counting error (25), and for sampling frequency (one in five sections, 150-μm intervals) by multiplying this product by a factor of five. Results thus represent estimates of the total number of Fos-positive cells per medial parvocellular region.

AVP hnRNA and CRH mRNA hybridization histochemistry. Acute activation of the PVN was independently assessed by examining within medial parvocellular neurons stress-induced changes in vasopressin transcriptional activation directly. This was achieved by means of an intron-specific cRNA probe directed at a series of evenly spaced sections through the rostrocaudal extent of the PVN, adjacent to the same tissue employed for Fos analysis. To further distinguish between potential gender and pubertal influences on steady-state and stress-induced CRH mRNA levels within neuronal and neurosecretory cell types, a cRNA probe encoding for CRH was directed at an additional parallel series through the PVN and extended to include the CeA.

Hybridization histochemistry was carried out using a [^{33}P]UTP-labeled (Amersham, Arlington Heights, IL) antisense cRNA probe transcribed from a 700-bp fragment of intron I of the rat vasopressin (26, 27) and a [^{33}P]UTP-labeled antisense cRNA probe transcribed from a full-length (1.2-kb) cDNA encoding CRH mRNA. Techniques for riboprobe synthesis, hybridization, and the patterns of hybridization for these probes in the PVN are described in greater detail elsewhere (28–30). Briefly, free-floating sections were first rinsed in KPBS to remove cryoprotectant and then mounted and vacuum dried on glass slides overnight. After postfixation with 10% formaldehyde for 30 min at room temperature, sections were digested in proteinase K (10 mg/ml, 37 C) for 30 min, acetylated for 10 min (2.5 mM acetic anhydride, 0.1 M triethanolamine, pH 8.0), rapidly dehydrated in ascending ethanol concentrations (50–100%), and then vacuum dried. Radionucleotide antisense cRNA probes were used at concentrations approximating 3×10^7 cpm/ml in a solution of 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, and 10 mM dithiothreitol, 1× Denhardt's solution, and 10% dextran sulfate and applied to individual slides. Slides were coverslipped and then incubated overnight at 57.5 C, after which the coverslips were removed and the sections washed three times in 4× standard saline citrate (SSC; 0.15 M NaCl, 15 mM citric acid, pH 7.0) at room temperature, treated with ribonuclease A (20 μg/ml) for 30 min at 37 C, desalted in descending SSC concentrations (2–0.1× SSC), washed in 0.1× SSC for 30 min at 60 C, and dehydrated in ascending ethanol concentrations. Hybridized sections were then exposed to x-ray film (β-max, Amersham), defatted in xylene, subsequently coated with Kodak NTB2 liquid autoradiographic emulsion, and exposed at 4 C in the dark with desiccant, the duration determined by the strength of signal on x-ray film (21 and 9 d for AVP hnRNA and CRH mRNA in the PVN, respectively; 12 d for CRH mRNA in the CeA). Slides were developed at 14 C with Kodak D-19 for 3.5 min, briefly rinsed in distilled water for 15 sec, fixed in Kodak fixer for 6.5 min, and then washed in running water for 45 min at room temperature.

Based on the strength of the autoradiographic signal, exposure time to emulsion was optimized to ensure that CRH mRNA and AVP hnRNA were within the linear range of the assay and could be quantified by making relative comparisons in OD levels. Analysis of the relative levels of vasopressin hnRNA and CRH mRNA in the PVN and CeA was performed on silver grain developed emulsion-coated slides using Macintosh-driven Open Lab Image Improvision software version 3.0.9 (Quorom Technologies). OD readings, corrected by background subtraction, were taken at regularly spaced (150-μm) intervals. Average OD values were determined bilaterally from three sections taken through the rostrocaudal extent of the medial parvocellular portion of the PVN and bilaterally from six sections through the CeA. Determination of CRH mRNA levels through a common rostrocaudal level of the amygdala was achieved by initiating densitometric analysis at 600 μm caudal to (or four sections from) the widest PVN expanse, as identified in an adjacent series of thionin-stained sections. Given the relatively dispersed nature of CRH-expressing neurons in the CeA, in addition to OD measures, CRH mRNA expression was further analyzed by counting the number of individual cell clusters. Only those nuclei identified as showing individual grain cluster densities of at least five times background were tallied. Numbers of CRH mRNA-expressing neurons were counted bilaterally and averaged through six sequential sections of the amygdala. To determine the potential by which gender and age differences in cell density could impact on the histochemical and hybridization data, stereological assessments of cell volume estimates (μm^2) were made using three and five adjacent thionin-stained sections through the medial parvocellular zone of the PVN and CeA, respectively.

Statistical analysis

Data are expressed as mean ± SEM and analyzed by using three- and two-way ANOVA as planned, to compare between- and within-sex differences in age × stress interactions, respectively. *Post hoc* analysis was performed, when appropriate, using Newman-Keuls for multiple pairwise comparisons and paired *t* test to compare effects of stress and age.

Results

Hormones

Basal gonadal and adrenal activity. Plasma testosterone, estrogen, progesterone, LH, and corticosterone concentrations were determined to validate pubertal age and/or shifts in basal gonadal and adrenal activity, respectively (Table 1). The 60-d-old male rats displayed significantly higher plasma levels of testosterone ($P < 0.0001$) and LH ($P < 0.01$) than 30-d-old male rats. Basal plasma progesterone and corticosterone concentrations were comparable between 30- and 60-d-old male rats. The 60-d-old females displayed significant elevations in plasma levels of estrogen, progesterone, and corticosterone relative to 30-d-old female rats ($P < 0.01$), although basal LH levels did not vary significantly ($P > 0.05$). Finally, there was no effect of stress on plasma testosterone ($P = 0.11$) and estrogen ($P = 0.123$) concentration in male and female rats, respectively.

Stress-related pituitary-adrenal activity. ACTH responses from baseline could not be assessed with confidence owing to the rapidity and variability by which the anterior pituitary corticotropes respond to chloral hydrate, reflected in the elevated prestress levels of plasma ACTH (Fig. 1). Nonetheless, a significant sex × age × stress interaction ($F_{(1,32)} = 4.5$; $P = 0.04$) was indicated. *Post hoc* analysis attributed this three-way interaction to effects under stress conditions, credited to 60-d-old males showing the lowest and 60-d-old females showing the highest plasma ACTH values at the termination of restraint ($P < 0.05$, pairwise comparisons).

Analysis of plasma corticosterone revealed significant effects of sex ($F_{(1,32)} = 19.8$; $P < 0.0001$), age ($F_{(1,32)} = 16.1$; $P = 0.0003$), stress ($F_{(1,32)} = 199.1$; $P < 0.0001$), and significant sex × age ($F_{(1,32)} = 7.9$; $P = 0.0083$), sex × stress ($F_{(1,32)} = 11.9$; $P = 0.0016$), and age × stress ($F_{(1,32)} = 5.7$; $P = 0.02$) interactions. As illustrated in Fig. 2, the bases for all of these interactions were the overwhelmingly higher basal and

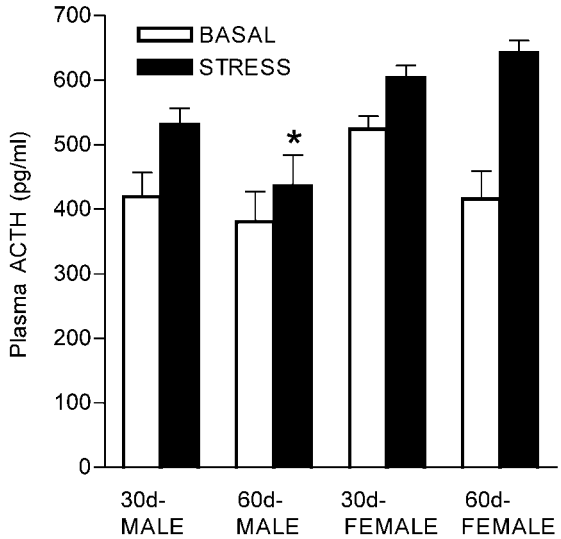

FIG. 1. Mean (± SEM) plasma ACTH concentrations (pg/ml) in 30- and 60-d-old male and female rats under basal conditions and at 30 min of restraint stress. *, $P < 0.05$ *vs.* pre- and postpubertal female stress values (n = 4 and n = 6 animals per group under basal and stress conditions, respectively).

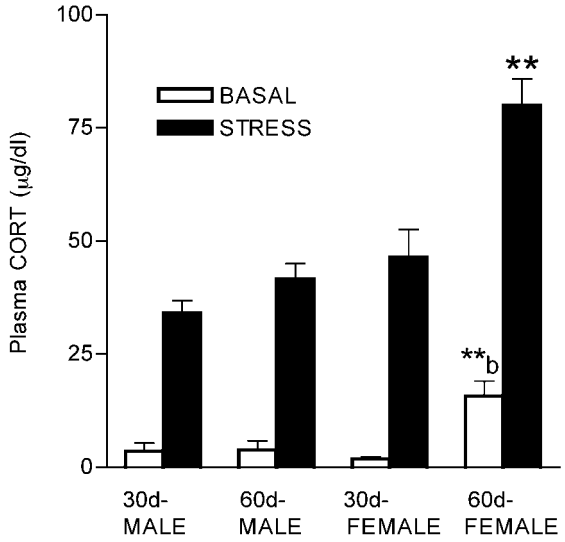

FIG. 2. Mean (± SEM) plasma corticosterone concentrations (μg/dl) in 30- and 60-d-old male and female rats under basal conditions and at 30 min of restraint stress. **b and **, $P < 0.01$ *vs.* basal and stress values, respectively (n = 4 and n = 6 animals per group under basal and stress conditions, respectively).

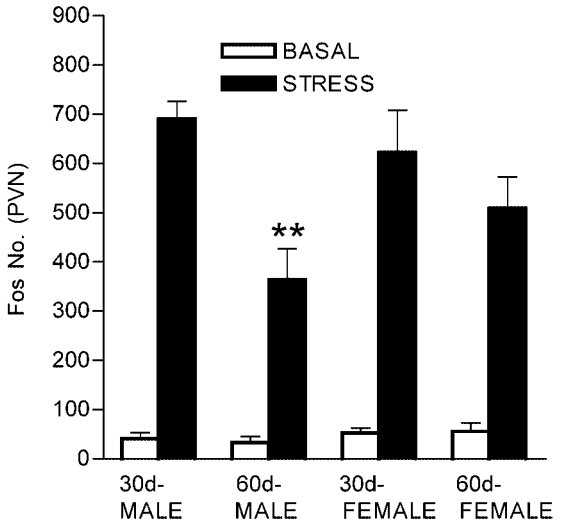

FIG. 4. Restraint-induced changes in the number of medial parvocellular Fos-ir cells in the PVN in 30- and 60-d-old male and female rats. **, $P < 0.01$ *vs.* male 30-d-old stress counterpart (n = 4 and n = 6 animals per group under basal and stress conditions, respectively).

stress plasma corticosterone concentrations in 60-d-old female rats.

Medial parvocellular activation

Fos induction. Quantitative assessment of the number of Fos-expressing medial parvocellular cells revealed significant effects of age ($F_{(1,32)} = 7.8$; $P = 0.0089$) and stress ($F_{(1,32)} = 152.7$; $P < 0.0001$) and a significant age × stress interaction ($F_{(1,32)} = 7.4$; $P = 0.01$). These effects were not attributable to differences in the basal state, as rats that did not experience restraint displayed few if any Fos-positive cells in the PVN (Fig. 3). *Post hoc* analysis indicated that the age × stress interaction was attributed to an age-related shift in male rats only (Fig. 4), produced by a significant decrement in the number of restraint-induced Fos-ir cells displayed by 60-d-old male rats ($P < 0.001$). Although responsive to restraint, female rats displayed no age-related change in the number of medial parvocellular neurons recruited to express Fos

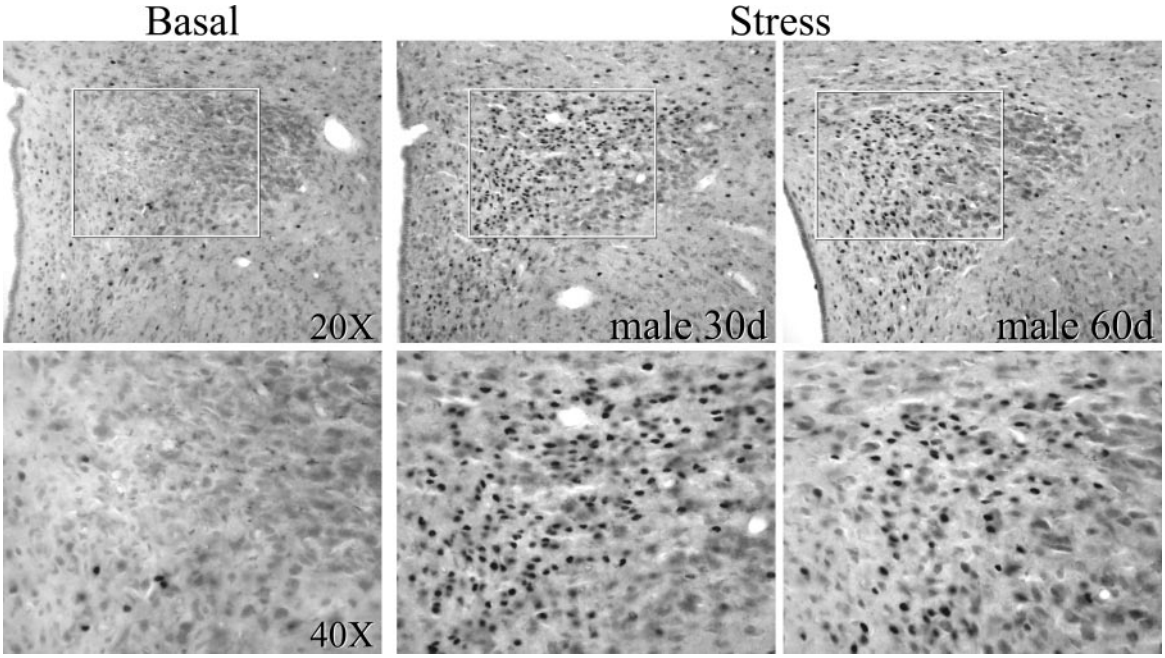

FIG. 3. Restraint-induced Fos expression in the PVN. Representative bright-field photomicrographs through the PVN under basal conditions (*left*) and displayed by 30- and 60-d-old male rats at 30 min of restraint stress (*middle* and *right*). Note the prevalence by which restraint induces Fos within cells occupying the mediodorsal portion of the PVN (*top*, ×20 magnification) and the inhibitory influence of puberty on the magnitude of Fos induction (*bottom*, ×40 magnification).

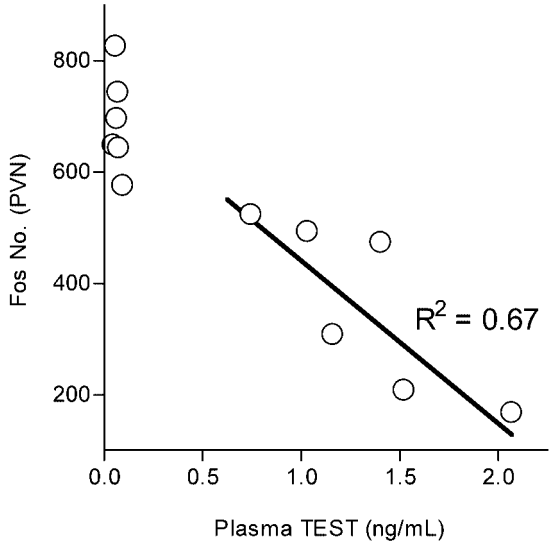

FIG. 5. Fos activation as a function of plasma testosterone concentration. Scattergram of the relationship between the total number of medial parvocellular neurons recruited to express Fos-ir and individual plasma testosterone levels at 30 min of restraint in 30- and 60-d-old male rats. (n = 12; six rats per stress/age group). Correlation and significance shown relate to 60-d-old rats only.

protein. Regression analysis in 60-d-old male rats revealed a reliable ($F_{(1,5)}$ = 7.9; P = 0.048) and negative relationship between the number of medial parvocellular neurons induced to express Fos protein and individual concentrations of plasma testosterone (Fig. 5).

AVP hnRNA induction. Densitometric analysis of AVP hnRNA expression within medial parvocellular nuclei (Fig. 6) revealed significant effects of sex ($F_{(1,32)}$ = 4.4; P = 0.04) and stress ($F_{(1,32)}$ = 54.7; $P < 0.0001$) and significant sex × puberty ($F_{(1,32)}$ = 4.3; P = 0.04) and sex × stress

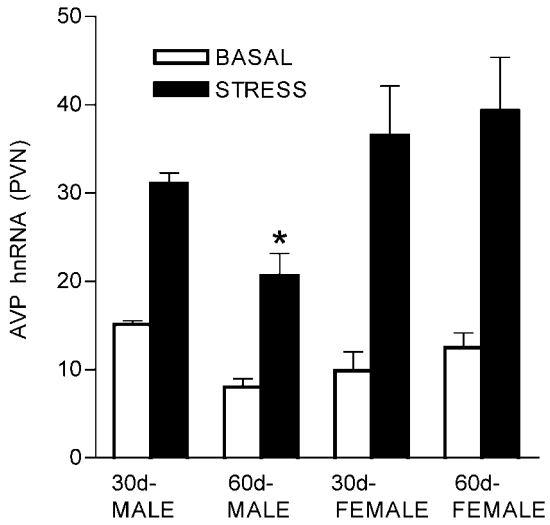

FIG. 7. Restraint-induced changes in medial parvocellular AVP gene expression in 30- and 60-d-old male and female rats. *, $P < 0.05$ *vs.* male 30-d-old stress counterpart (n = 4 and n = 6 animals per group under basal and stress conditions, respectively).

($F_{(1,32)}$ = 5.0; P = 0.03) interactions. *Post hoc* analysis indicated that the sex × stress interaction was produced by sex differences in AVP transcription induced by stress ($F_{(1,20)}$ = 7.8; P = 0.01). This sex difference in AVP hnRNA expression, as revealed by within-sex, two-way analysis (Fig. 7), was credited to an age-related effect in males ($F_{(1,16)}$ = 23.9; $P < 0.0002$) but not in females ($F_{(1,16)}$ = 0.265; P = 0.61). Regression analysis in 60-d-old male rats revealed a reliable ($F_{(1,5)}$ = 21.1; P = 0.0101) and negative relationship between stress-induced AVP transcriptional activation and individual concentrations of plasma testosterone (Fig. 8).

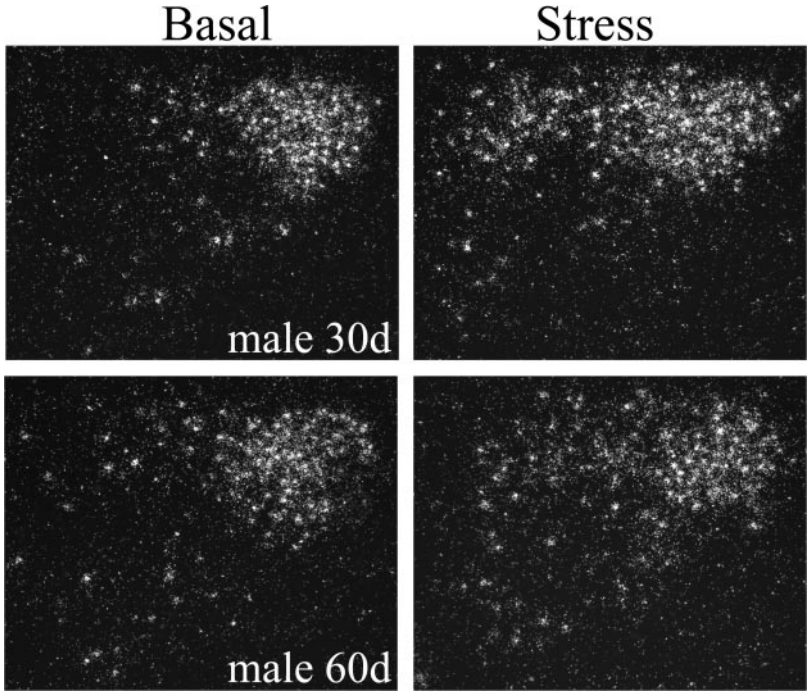

FIG. 6. Stress and pubertal influences on AVP gene transcription in males. Representative dark-field photomicrographs of AVP hnRNA expression under basal and stress conditions (*left* and *right*) displayed by 30- and 60-d-old male rats (*top* and *bottom*). Note the prevalence of AVP hnRNA within the posterior magnocellular neurons under basal conditions and the induced transcription within the hypophysiotropic zone of the PVN in response to restraint.

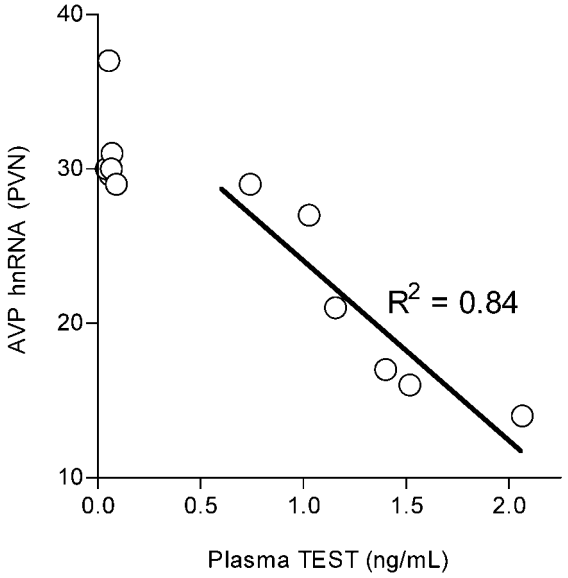

FIG. 8. AVP gene activation as a function of plasma testosterone concentration. Scattergram of the relationship between restraint-induced levels of AVP hnRNA expression and individual plasma testosterone levels at 30 min of restraint in 30- and 60-d-old male rats (n = 12; six rats per stress/age group). Correlation and significance shown relate to 60-d-old rats only.

CRH mRNA expression

Paraventricular nucleus. A series of sections through the medial parvocellular portion of the PVN (Fig. 9) and CeA (Fig. 10) was used to determine age- and sex-related differences in CRH mRNA levels by hybridization densitometry. At the level of the PVN, three-way ANOVA revealed significant effects of sex ($F_{(1,32)} = 4.8$; $P = 0.034$), age ($F_{(1,32)} = 4.5$; $P = 0.042$), and stress ($F_{(1,32)} = 9.0$; $P = 0.0051$) and a three-way interaction that approached significance ($F_{(1,32)} = 3.9$; $P = 0.054$). Illustrated in Fig. 11, *post hoc* analysis indicated that the sex and age effects were attributed to higher basal CRH mRNA levels in the PVN in 60-d-old female rats ($P < 0.05$; basal pairwise comparisons). Within-sex, two-way analysis showed no effect of stress on medial parvocellular CRH mRNA levels among females ($F_{(1,16)} = 1.9$; $P = 0.19$), although a significant positive effect was revealed in 60-d-old males ($F_{(1,16)} = 12.5$; $P = 0.002$).

Central amygdala. Three-way ANOVA revealed significant effects of sex ($F_{(1,32)} = 15.4$; $P = 0.0004$) and age ($F_{(1,32)} = 65.1$; $P < 0.0001$) and a significant sex × age interaction ($F_{(1,32)} = 16.7$; $P = 0.0003$) on CRH mRNA expression in the CeA (Figs. 10 and 12). The sex × age interaction was attributed to an age-related increase in male rats only ($P < 0.01$). Finer-grain analysis indicated that this densitometric elevation in CRH expression displayed in 60-d-old male rats was produced by an increase in the number of CRH mRNA-expressing cells (Fig. 12). Stereological estimates indicated a comparable age-related increase in CeA volume in male and female rats (data not shown), indicating that cell density does not contribute to the sex difference in the number of CRH-expressing cells displayed by 60-d-old rats.

Discussion

Plasma hormones revealed major age-related shifts in basal gonadal and adrenal status in both male and female rats (Table 1). The 60-d-old male and female rats displayed significant elevations in plasma sex steroid concentrations compared with their 30-d-old counterparts. Female, but not male, rats showed an age-related increase in basal corticosterone release. All told, these findings are consistent with previous studies identifying pubertal shifts in gonadal function (20–23, 31) and the emergence of a sex difference in basal plasma corticosterone and adrenal function (32–35). In addition to

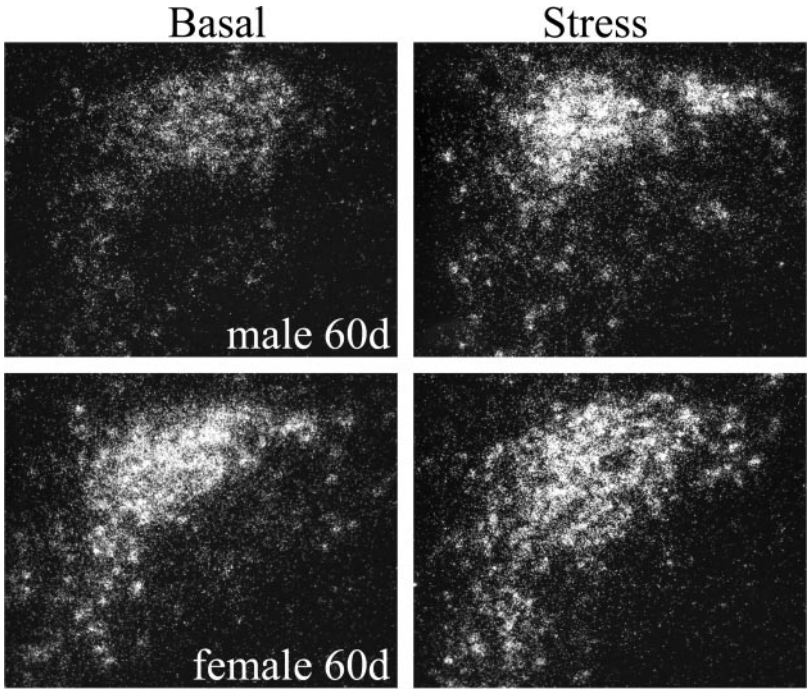

FIG. 9. Sex and stress interact on medial parvocellular CRH. Representative dark-field photomicrographs of CRH mRNA expression in 60-d-old male and female rats (*top* and *bottom*) under basal and stress conditions (*left* and *right*). The 60-d-old females displayed relatively higher CRH mRNA levels under basal conditions, and 60-d-old males showed restraint-induced elevations in CRH mRNA.

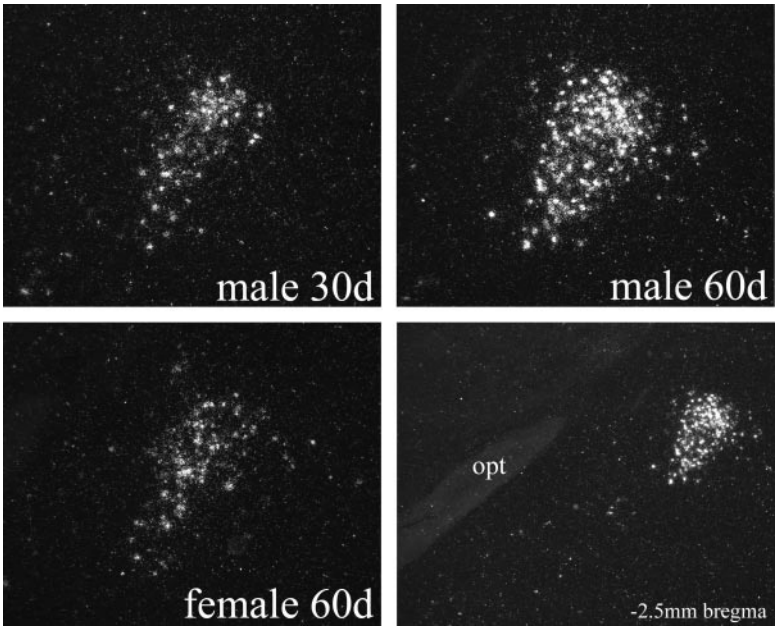

FIG. 10. Sex and puberty interact on basal CRH expression in the CeA. Representative dark-field photomicrographs showing the relative expression levels of CRH mRNA in 30- and 60-d-old male rats (*top*) and in 60-d-old female rats under basal conditions (*bottom left*); ×20 magnification. The view under ×10 magnification (*bottom right*) shows the rostral extent of CRH-expressing cells in the CeA (*opt*, optic tract).

validating the age groups employed with respect to puberty, the sex steroids assayed provided a means of correlating, when applicable, individual differences in HPA activity as a function of gonadal status.

In males, stress-induced levels of corticosterone at 30 min of restraint in 60-d-old rats were comparable to 30-d-old rats (Fig. 2). This does not refute a pubertal influence on stress HPA activity in males. As previously shown, pubertal and testicular influences on the adrenal response are most evident at poststress intervals, in keeping with an inhibitory effect of testosterone on stress-induced HPA activation (14, 15). Shifts in stress HPA activity were more readily apparent in females, in which plasma corticosterone responses were highest overall in 60-d-old female rats. Finally, restraint-induced ACTH levels were highest in 60-d-old females and lowest in 60-d-old males (see *Results*), consistent with a stimulatory shift in females and an inhibitory shift in males in HPA stress responsiveness.

Given this dissociation in HPA output in adult male and female rats leaving puberty, we expected to see marked age- and sex-related differences in the stress-induced activation of medial parvocellular PVN neurons. Relative to their 30-d-old counterparts, 60-d-old males displayed a significant reduction in the number of medial parvocellular neurons recruited to express Fos protein and a reduction in stress-induced AVP transcription (Fig. 4). In males, both of these independent markers of synaptic and transcriptional activation varied strongly and negatively with testosterone (Figs. 5 and 8). Most impressive was the fact that these markers varied sig-

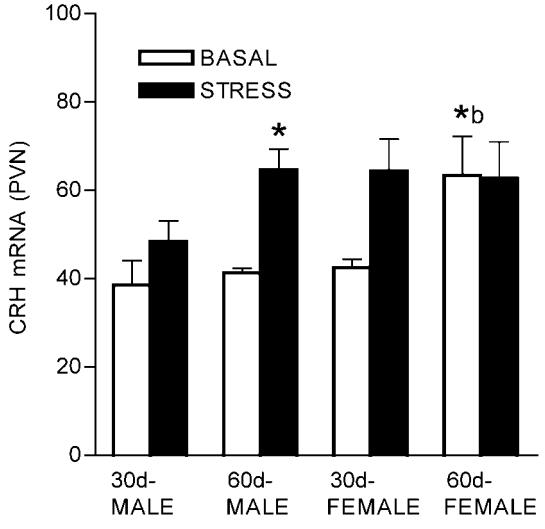

FIG. 11. Relative expression levels of medial parvocellular CRH mRNA in 30- and 60-d-old male and female rats under basal conditions and at 30 min of restraint stress. *, $P < 0.05$ *vs.* male 30-d-old stress counterpart; *b, $P < 0.05$ *vs.* all basal values (n = 4 and n = 6 animals per group under basal and stress conditions, respectively).

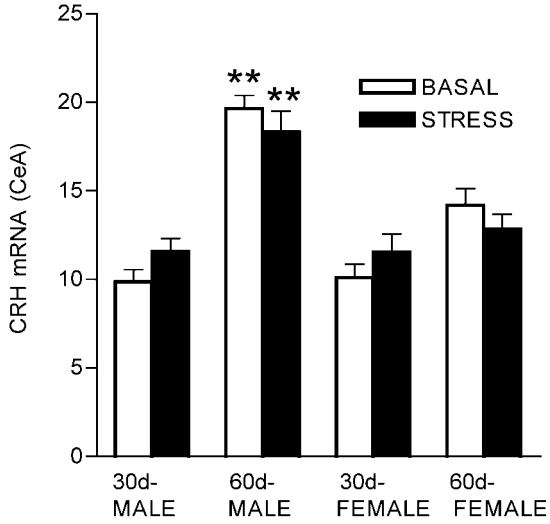

FIG. 12. Relative CRH mRNA expression levels in the CeA in 30- and 60-d-old male and female rats under basal conditions and at 30 min of restraint stress. *, $P < 0.05$ *vs.* 30-d-old males (n = 4 and n = 6 animals per group under basal and stress conditions, respectively).

nificantly as a function of testosterone within the 60-d-old group but not in the 30-d-old male group. Consistent with our previous studies, plasma testosterone levels were not significantly altered by acute restraint exposure (15). We can assume, therefore, that the variability in testosterone reflects naturally occurring differences in gonadal status existing before restraint exposure (reviewed in Ref. 3, and see Refs. 36 and 37). Plasma ACTH and parvocellular Fos and AVP hnRNA responses to restraint vary strongly and negatively as a function of basal, prestress levels of testosterone in intact and testosterone-replaced gonadectomized adult male rats (3, 15). Taken together with our current findings, this suggests that pubertal shifts in stress responsiveness in males are explained by testosterone and that the dose-related inhibitory effects of testosterone are enlisted once the HPA axis matures. This is consistent with the finding that testosterone is less effective in reducing the corticosterone response to restraint in 28-d-old rats than in 77-d-old rats (14).

In contrast, despite showing higher inducible levels of AVP hnRNA and a shift in the magnitude of the ACTH and corticosterone responses to stress, medial parvocellular neurons in females displayed no age-related shift in stress-induced Fos and AVP transcriptional activation (Figs. 4 and 8). This incongruity could be explained, in part, by a greater consumption of CRH, verifiable by an elevation in stress-induced CRH transcription in adult female rats, provided this transcriptional activation reflects the mechanism to initiate replenishment of peptide stores. Medial parvocellular Fos mRNA induction does not vary across the estrous cycle in response to restraint (38) but does vary in response to immune challenge (39). The Fos response to endotoxin is elevated during proestrous when plasma estrogen concentrations are highest. Thus, medial parvocellular neurons in female rats have the capacity to express Fos in a manner that parallels HPA output and gonadal status, although the extent of this influence appears stress specific.

Alternatively, differences in stress HPA activity between pre- and postpubertal females may not necessarily require an alteration in neural input evoking ACTH secretagogue release. The capacity to respond to restraint and pubertal influences thereof could be met by alterations in the drive to synthesize ACTH secretagogues. Basal CRH mRNA expression levels within medial parvocellular neurons were significantly higher in 60-d-old than in 30-d-old female rats (Fig. 11). Thus, pubertal elevations in pituitary-adrenal responsiveness in females may be credited to a parallel elevation in hypophysiotropic CRH synthesis. In contrast, male rats displayed no age-related shift in basal CRH mRNA in the PVN, indicating, yet again, that the brunt of the pubertal alterations in males in HPA function appear to rely on changes in stress-induced neuronal activation rather than steady-state alterations in peptide expression. Along similar lines, acute stress provoked an increase in CRH mRNA in 60-d-old but not in 30-d-old male rats (Fig. 11). This also suggests in older male rats an increased capacity of parvocellular neurons to use CRH. The extent to which this reflects a pubertal or testicular influence independent of testosterone remains unclear, given the apparent selectivity by which testosterone operates on AVP but not CRH expression within medial parvocellular PVN neurons.

Sex- and age-dependent differences in basal CRH mRNA expression were clearly indicated within the CeA. Relative to 30-d-old male rats, 60-d-old males displayed higher levels of CRH mRNA, produced by greater numbers of CRH-expressing cells (Figs. 10 and 12). Female rats on the other hand, showed no such age-related change in CRH expression. Adult male rats display an increase in CRH mRNA in the CeA after castration, and this effect is reversed with testosterone replacement (unpublished observation). Although indicative of an inhibitory influence in adult males, this disputes a role for testosterone in mediating the basal elevation in CRH expression in the CeA associated with puberty.

The primary intention of this study was to shed light on the nature by which stress HPA activity changes as a function of puberty in males. Females were employed as a reference, because the magnitude of the HPA response sexually differentiates at puberty (25, 27). Thus, we expected the bulk of the sex differences to be quantitative rather than qualitative. As we discovered, pubertal differences were emphasized by changes in synaptic and transcriptional activation in males and by changes in CRH synthesis in females, as suggested by steady-state alterations in basal CRH mRNA expression in the PVN.

The strength to which these sex differences can be attributed to puberty or otherwise confounded by cyclic variations in adult females warrants some scrutiny, as corticosterone secretion and CRH mRNA levels in the PVN vary over the estrous cycle (40, 41). The amplitude of this variance, however, does not approach the range of the age- and sex-related differences identified (see Ref. 42), playing down an influence of the estrous cycle on our findings. Furthermore, as discussed earlier, restraint-induced Fos mRNA expression in the PVN does not vary over the estrous cycle (38). Thus, the puberty-related shift in parvocellular activation remains unique to males and tenable to a pubertal influence.

One prevalent assumption is that both sexes use the same stress transduction system (see Ref. 43). However, our present findings indicate that both the extent and manner by which the ACTH secretagogues CRH and AVP are used and subject to pubertal influences are completely different. One basis for this differentiation can be tied to the selective and differential effects of testosterone on AVP and estrogen on medial parvocellular CRH (4, 5, 15). This regulation would have to be placed distal to or upstream from the PVN. The androgen receptor and the estrogen β-receptor isoform are prevalent within the PVN. However, in both sexes they are distributed almost to the exclusion of the ACTH-regulating, median eminence-projecting PVN neurons, and contained by preautonomic brain stem- and spinal-projecting cells (3, 44, 45). Fittingly perhaps, candidate forebrain structures integrating pubertal-gonadal interactions on HPA function could reside within the medial preoptic nuclei, central and medial nuclei of the amygdala, and stria terminalis bed nuclei. All of these contain androgen and estrogen receptors, project directly or indirectly to the PVN, relay homeostatic information, and regulate reproduction (3). Although approaching how these structures mediate and integrate gonadal-adrenal interactions during puberty is a formidable task, the signature responses in the PVN as revealed here, however, provide a framework for studying the relative con-

tribution and downstream effects of these candidate nuclei on medial parvocellular function (3, 10, 46).

At stake is the larger question of what the changes in the PVN and in the amygdala mean to puberty. Studies examining the adaptive value of pubertal shifts in ovarian status on HPA function in females are lacking. In male rats transiting puberty, the manner by which the HPA axis meets and protects against the metabolic demands of stress rests with the capacity to synthesize testosterone and to alter the pattern of testosterone release in the face of stress. Thus, as Gomez and Dallman (8) discovered, when plasma testosterone levels are clamped by static testosterone replacement, prepubertal but not postpubertal male rats are more adept in mounting a corticosterone response and surviving cold stress. Thus, the burden of a successful emergence from puberty in males would fall on the ability of the HPA axis to acquire and respond to variations in plasma testosterone concentrations.

Key to this is the dose-related manner by which the stress-induced transcriptional activation of medial parvocellular AVP responds to testosterone replacement (5, 15) and, as shown here, its variation as a consequence of puberty. Moreover, in adult male rats, alterations in the magnitude of the ACTH and corticosterone response to stress vary more strongly with changes in AVP than with medial parvocellular CRH activity (47–49). Although requiring further study, our findings suggest that relative to males, the onus in females could be to acquire a greater and timely capacity to synthesize CRH. To this end, a wide and species-broad literature exists with respect to the precocious effects of stress and/or premature adrenarche on the timing of sexual maturation in females (50–53), although the long-term effects of this on HPA plasticity has not been systematically approached.

Puberty is a dynamic period that is characterized by marked changes in the activity and connectivity of the amygdala, hippocampus, and mesocorticolimbic systems, thought to promote or program effective behavioral (avoidance learning and novelty seeking), autonomic (cardiovascular and metabolic), and neuroendocrine (adrenal, gonadal, and metabolic) responses to increased and new environmental demands (54–58). Although these changes are adaptive, they may also place the individual at risk considering the proclivity by which adolescents succumb to mental disorders over the course of puberty (59–61). The puberty-related increase in CRH mRNA expression in the CeA displayed by males speaks to this possibility, considering the role that CRH in the amygdala normally plays in mediating fearfulness and vigilance, but anxiety and behavioral hyperreactivity under more chronic stress conditions. Interestingly, repeated restraint-induced changes in CRH immunoreactivity in the amygdala are antagonized by androgen receptor blockade in 60-d-old but not in 40-d-old male rats (10). Perhaps this dependence on testosterone affords a greater capacity for older animals to adapt. Conversely, whether this independence from testosterone during early puberty in younger males explains their increased vulnerability to stress remains an intriguing question.

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