

Stress History and Pubertal Development Interact to Shape Hypothalamic-Pituitary-Adrenal Axis Plasticity

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Both the magnitude and the duration of the hormonal stress response change dramatically during neonatal development and aging as well as with prior experience with a stressor. However, surprisingly little is known with regard to how pubertal maturation and experience with stress interact to affect hypothalamic-pituitary-adrenal axis responsiveness. Because adolescence is a period of neurodevelopmental vulnerabilities and opportunities that may be especially sensitive to stress, it is imperative to more fully understand these interactions. Thus, we examined hormonal and neural responses in prepubertal (28 d of age) and adult (77 d of age) male rats after exposure to acute (30 min) or more chronic (30 min/d for 7 d) restraint stress. We report here that after acute stress, prepubertal males exhibited a significantly prolonged hormonal stress response (e.g. ACTH and total and free cortico-

sterone) compared with adults. In contrast, after chronic stress, prepubertal males exhibited a higher response immediately after the stressor, but a faster return to baseline, compared with adults. Additionally, we demonstrate that this differential stress reactivity is associated with differential neuronal activation in the paraventricular nucleus of the hypothalamus, as measured by FOS immunohistochemistry. Using triple-label immunofluorescence histochemistry, we found that a larger proportion of CRH, but not arginine vasopressin, cells are activated in the arginine vasopressin response to both acute and chronic stress in prepubertal animals compared with adults. These data indicate that experience-dependent plasticity of the hypothalamic-pituitary-adrenal neuroendocrine axis is significantly influenced by pubertal maturation. (*Endocrinology* 147: 1664–1674, 2006)

IN RESPONSE TO STRESSORS, the hypothalamic-pituitary-adrenal (HPA) axis is driven by the release of CRH and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus (PVN). These neuropeptides then allow for the release of ACTH from the anterior pituitary, which, in turn, stimulates the release of corticosterone from the adrenals (1). In the short term, this response allows the animal to cope with the immediate demands imposed by the stressful event. However, longer or more chronic exposures to stress hormones can result in a number of negative effects, particularly with regard to neurobiological function (2).

Throughout an individual's life span, both the magnitude and the duration of the hormonal stress response change dramatically. For instance, neonates show reduced stress reactivity in response to stressors that typically elicit robust stress responses in adults (3). Conversely, aged adults show heightened and more prolonged stress responses compared with younger adults (4). The reduced stress reactivity experienced by neonates has been posited to protect the devel-

oping organism from the damaging effects of stress hormones (3), whereas the extended exposure to stress hormones in the aged may contribute to the age-related decline in neurophysiological function and contribute to fat deposition, bone mineral loss, and impaired immune function in the elderly (4–6). Thus, parameters that change stress reactivity, such as development, may have profound consequences as to whether stressors lead to adaptive or maladaptive responses.

Although stress responsiveness in neonatal and adult life stages has been well characterized, little is known about how stress reactivity changes during puberty. This is interesting, because many brain regions implicated in the control of the stress response, including the hypothalamus, hippocampus, amygdala, and prefrontal cortex, continue to mature during pubertal development (7–11). The few studies comparing HPA reactivity in prepubertal and adult animals show that in response to acute stress, prepubertal animals have a significantly prolonged hormonal stress response compared with adults (12–15).

In addition to development, experience with stressors can influence stress reactivity. For instance, in adults, repeated exposure to a stressor leads to habituation of the stress response, such that stress hormone levels are blunted (16–19). Whether experience with stress changes HPA reactivity in prepubertal animals is unknown. Thus, in the present experiments we examined hormonal and neural responses in prepubertal and adult males after exposure to either acute (30 min) or more chronic (30 min/d for 7 d) restraint stress. We report in this study that pubertal development and stress

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Abbreviations: AcbSh, Nucleus accumbens shell; AVP, arginine vasopressin; CBG, corticotropin-binding globulin; CeA, central nucleus of the amygdala; Exp, experiment; HPA, hypothalamic-pituitary-adrenal; ir, immunoreactive; mPFC, medial prefrontal cortex; PB, phosphate buffer; PVN, paraventricular nucleus of the hypothalamus; TB, Tris buffer; vLS, ventral lateral septum.

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history interact to modulate both the immediate poststress response and recovery to baseline after termination of the stressor. Furthermore, this differential stress responsiveness is associated with greater activation, of CRH, but not AVP, neurons (as measured by FOS immunostaining) in the PVN of prepubertal compared with adult males. Together, these data indicate that experience-dependent plasticity of the HPA neuroendocrine axis is significantly influenced by pubertal development, and that CRH neurons of the PVN are at least one neural locus involved in mediating these changes.

Materials and Methods

Animals and housing

For all experiments, male Sprague Dawley rats were commercially obtained from Charles River Laboratories (Harlan, NY), housed three per cage (same age and poststress time point cage mates) in clear polycarbonate cages with wood chip bedding, and maintained on a 12-h light, 12-h dark schedule (lights on at 0900 h). Prepubertal and adult animals arrived on the same day. Prepubertal animals were weaned from their mothers on the day of arrival (20 d of age) and evenly distributed among the groups. All animals had *ad libitum* access to food and water, and the vivarium was maintained at 21 ± 2 C. All procedures were carried out in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Experimentation Guidelines from the committee on animal research of Rockefeller University.

Experimental design and tissue processing

Four experiments were conducted. In experiments (Exp) 1.1 and 1.2, prepubertal (28 d of age) and adult (77 d of age) male rats were exposed to a single 30-min session of restraint stress (acute stress), and hormonal (Exp 1.1) and neural (Exp 1.2) responses were measured. In Exp 2.1 and 2.2, prepubertal (22 d of age) and adult (70 d of age) males were exposed to 7 consecutive days of a 30-min session of restraint stress (chronic stress). On the seventh day, hormonal (Exp 2.1) and neural (Exp 2.2) responses were measured (*i.e.* at either 28 or 77 d of age). In these studies, 28-d-old males were defined as prepubertal because Sprague Dawley rats at this age exhibit testosterone levels well below those found in adults (Refs. 14, 20, and 21 and present study) and do not show appreciable pubertal increases in testosterone secretion before 35 d of age (20).

For Exp 1.1, prepubertal (28 d) and adult (77 d) animals were weighed and rapidly decapitated by guillotine either before (basal) or after a 30-min session of restraint stress. Two time points after the stressor were examined: immediately after termination of the stressor (*i.e.* time zero) and 45 min after the stress session (six animals per age and time point). The 45-min point was chosen to assess recovery from the stressor and was based on previous studies indicating that prepubertal animals show a higher stress response compared with adults at this time point (12–15).

Animals were restrained in wire mesh restrainers, sized so that animals were equally immobilized and thus exposed to equivalent stressors. Upon termination of the stressor, animals were either immediately decapitated or returned to their home cage until the appropriate time point (*i.e.* 45 min). Trunk blood samples were collected in Vacutainer K₃ EDTA-coated test tubes (BD Biosciences, Franklin Lakes, NJ) and spun down at 4 C in a refrigerated centrifuge. All animals were killed between 1200–1500 h local time during their circadian nadir of corticosterone secretion to minimize variations in basal hormone levels (22). Plasma was removed and stored at -20 C until the RIAs for ACTH, corticosterone, corticotropin-binding globulin (CBG), and testosterone were performed (see below). Testes and adrenal glands were removed, cleaned of fat, and weighed to verify developmental stage.

For Exp 1.2, prepubertal (28 d) and adult (77 d) males were treated in an identical manner as described above (five animals per age and time point), but instead of decapitation on the day of sampling, all animals were perfused after an overdose of sodium pentobarbital (100 mg/kg) to generate tissue for immunohistochemistry. Animals were transcardially perfused with 150 ml 0.9% heparinized saline, followed by 200 ml

4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were post-fixed for 4 h in 4% paraformaldehyde, then stored in 30% sucrose at 4 C. Coronal brain sections were made on a Vibratome (Leica VT 1000S; Wetzlar, Germany) (40 μ m) and stored at -20 C in cryoprotectant until processed for immunohistochemistry (see below).

In Exp 2.1, prepubertal (22 d) and adult (70 d) males were exposed to chronic stress (30 min/d for 7 d) and decapitated, and blood samples were collected on the seventh day of stress (*i.e.* at either 28 or 77 d of age). The same stress procedure was used, and the same time points were measured as in Exp 1.1 (six animals per age and time point). In Exp 2.2, prepubertal (22 d) and adult (70 d) animals were treated in an identical manner as those in Exp 2.1 (five per age and time point), but on the day of sampling (*i.e.* at either 28 or 77 d of age), animals were perfused to generate tissue for immunohistochemistry.

RIAs

ACTH, corticosterone, and testosterone assays were conducted on the trunk blood samples collected in Exp 1.1 and 2.1 using commercially available kits and reagents and were performed as indicated by the supplier. ACTH measurements were obtained with an ACTH ¹²⁵I RIA Kit (DiaSorin, Stillwater, MN) using the overnight incubation protocol (option A), whereas corticosterone and testosterone measurements were made using Coat-A-Count kits (Diagnostic Products Corp., Los Angeles, CA). For each hormone, all samples were run in duplicate in a single assay, and values were averaged. The lower limit of detectability and intraassay coefficient of variation for each assay were as follows: ACTH, 10.02 pg/ml and 8.4%; corticosterone, 9.69 ng/ml and 6.5%; and testosterone, 0.02 ng/ml and 12.6%.

For CBG quantification, a radioligand binding assay was performed as previously described (23, 24). Briefly, plasma was diluted in an equal volume of 1% charcoal and 1% dextran-coated charcoal/Tris buffer [50 mM Tris-0.1% Norit-A charcoal (pH 7.4)] to eliminate steroids from plasma. After a 10-min incubation, the suspension was centrifuged at $2000 \times g$ (10 min at 4 C), and a portion of the resulting supernatant (stripped plasma) was further diluted 1:66.67 with Tris buffer (TB; pH 7.4). A radioligand binding assay for CBG was conducted in triplicate by incubating 50 μ l diluted plasma with 50 μ l [1,2,6,7-³H]corticosterone (specific activity, 88 Ci/mmol; NEN Life Science Products, Boston, MA) and 50 μ l TB (final dilution, 1:800) for 1 h. Free and bound [³H]corticosterone were separated by rapid filtration over Whatman GF/B glass-fiber filters (Clifton, NJ) that were soaked for 1 h in cold TB containing 0.3% polyethylenimine (Sigma-Aldrich Corp., St. Louis, MO). After filtration, filters were immediately rinsed three times with ice-cold TB and placed into liquid scintillation glass vials. Filter-bound radioactivity was quantified using a standard liquid scintillation counter (LS6500, Beckman Instruments, Fullerton, CA). Separate nonspecific binding tubes were also assayed (50 μ l competitor plus 50 μ l [³H]corticosterone). All samples were assayed in triplicate, and median value was used for analyses. Free corticosterone was derived from CBG values as described by Barsano and Baumann (25). Specifically, free corticosterone was calculated as follows:

$$0.5 \left[\text{nM CORT} - \text{nM CBG} - K_d \right. \\ \left. + \sqrt{(\text{nM CBG} - \text{nM CORT} + K_d)^2 + 4(\text{nM CORT}/(1/K_d))} \right],$$

where CORT is corticosterone.

Single-labeled FOS immunohistochemistry

Tissue from Exp 1.2 and 2.2 was processed for FOS immunostaining to measure stress-induced changes in neuronal activation in prepubertal and adult males exposed to either acute or chronic stress. FOS immunoreactivity is a widely used technique to assess stress-induced neuronal activation in response to a variety of stressors, including restraint (21, 26–31). To minimize potential variability and achieve optimal staining, each brain region in the acute and chronic groups was processed simultaneously. Free-floating sections (40 μ m) were washed five times for 10 min each time in 0.1 M PB and incubated for 10 min in 0.05% H₂O₂ in 0.1 M PBS. Sections were then washed three times for 10 min each time in 0.1 M PB with 0.1% Triton X-100 (PBT), blocked in 2% normal goat serum in PBT for 1 h, and incubated in rabbit anti-Fos (1:20,000; Santa

Cruz Biotechnology, Inc., Santa Cruz, CA) in 2% normal goat serum in PBT for 48 h at 4°C. Sections were then washed five times for 10 min each time in PBT and incubated in biotinylated goat antirabbit IgG (1:200; Vector Laboratories, Inc., Burlingame, CA) in PBT for 1 h at room temperature. Sections were washed three times for 10 min each time in PBT and incubated in avidin-biotin horseradish peroxidase complex (1:200; Vectastain ABC Kit, Vector Laboratories, Inc.) in PBT for 1 h at room temperature. After three 10-min washes in PBS, horseradish peroxidase was visualized with 0.02% 3,3'-diaminobenzidine in a 3-M sodium acetate buffer containing 2.5% nickel sulfate and 0.05% H₂O₂. Sections were then washed five times for 10 min each time in PBS, mounted onto Fisher Brand Plus slides (Fisher Scientific, Pittsburgh, PA), dried, dehydrated in increasing concentrations of alcohol (70%, 95%, and 100%), cleared in xylenes, and coverslipped with DPX Mountant (Sigma-Aldrich Corp.). Processing tissue in the absence of the primary antibody resulted in no detectable immunostaining by the secondary antibody.

Triple-labeled FOS/CRH/AVP immunofluorescence histochemistry

A randomly chosen subset of animals (three or four per age and time point) that had been previously used in the single-labeled FOS experiments were processed for triple-label immunofluorescence histochemistry to quantify cell number and colocalization of FOS, CRH, and AVP in the PVN in prepubertal and adult animals exposed to either acute or chronic stress. Again, to minimize potential variability, all PVN sections from animals in either the acute or chronic groups were run simultaneously and were anatomically matched. For triple-label immunofluorescence, free-floating sections (40 μ m) were washed five times in PBT for 10 min each time and incubated in normal donkey serum for 1 h, then in goat anti-Fos (1:5000; Santa Cruz Biotechnology, Inc.), rabbit anti-CRH (1:5000; Peninsula Laboratories, Inc., Belmont, CA), and guinea-pig anti-AVP (1:5000; Peninsula Laboratories, Inc.) simultaneously for 48 h. Sections were washed three times in PBT for 10 min each time, then placed in the appropriate donkey secondary antibody conjugated to the CY-2, CY-3, and CY-5 fluorescent chromogens (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. Sections were washed three times for 10 min each time in PB, mounted on gelatin-coated slides, dried, dehydrated in increasing concentrations of ethanol (70%, 95%, and 100%), cleared in xylenes, and coverslipped with Krystalon (EM Science, Gibbstown, NJ). Processing tissue in the absence of the primary antibodies resulted in no detectable immunostaining by the appropriate secondary antibody.

Microscopic analysis of single-labeled FOS immunohistochemistry

The areal density (cells per unit area) of FOS-immunoreactive (FOS-ir) cell profiles was quantified in the infralimbic cortex of the medial prefrontal cortex (mPFC), nucleus accumbens shell (AcbSh), ventral lateral septum (vLS), PVN, and central nucleus of the amygdala (CeA). These areas were chosen for examination because they have been demonstrated to display stress-induced increases in FOS expression in adult rats (32) and to play important roles in modulating HPA axis activity (*e.g.* mPFC, CeA, and PVN) (1, 33). Brain sections were inspected under light microscopy using a Labophot microscope (Nikon, Melville, NY). Three sections through each nucleus, separated by 120 μ m and anatomically matched across animals, were used for analysis. Each brain region was

centered in the field of view at $\times 4$, and the magnification was increased to $\times 20$. At least two bilateral counts were made for each brain area. A cell was considered immunopositive when dark blue reaction product was observed in the nucleus. All data are expressed as mean number of FOS-ir cells per 40,000 μ m². One experimenter, blind to the experimental conditions of the animals, was responsible for FOS-ir areal density measures.

Confocal microscopy and analysis of FOS/CRH/AVP immunofluorescence histochemistry

Confocal microscopy was used to examine the cell number and colocalization of FOS, CRH, and AVP with a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, NY) and a Zeiss LSM 510 META scanning confocal attachment. Sections were excited sequentially with an argon-krypton laser using the standard excitation wavelengths for CY-2, CY-3, and CY-5. Stacked images were collected as 1- μ m multitract optical sections. LSM 3.95 software (Carl Zeiss) was used to superimpose CY-2 (green), CY-3 (red), and CY-5 (blue) images of the sections. To evaluate the extent of colocalization, each channel was observed and quantified independently. Channels were then combined, and double- or triple-labeled cells were identified. Two bilateral counts from anatomically matched sections were made for each animal. Each section of the PVN was examined in its entirety in 1- μ m steps. One experimenter, who was blind to the experimental conditions of the animals, was responsible for confocal analyses.

Statistical analysis

For the four experiments, all parametric data were analyzed using a two-way ANOVA (age \times time point), whereas percentage data were first arcsine transformed, then analyzed using two-way ANOVAs. Significant main effects and interactions were analyzed with Tukey's honestly significant difference tests. Differences were considered significant at $P < 0.05$. All data are reported as the mean \pm SEM. For presentation purposes, hormonal data (Exp 1.1 and 2.1) are presented first, followed by the neural data (Exp 1.2 and 2.2).

Results

Experiential and developmental interactions on the hormonal stress response

Peripheral measures. In the acute stress groups (Exp 1.1), there were significant main effects of age on body [F(1,30) = 3740.553; $P < 0.05$], testis [F(1,30) = 1812.232; $P < 0.05$], and adrenal [F(1,30) = 107.580; $P < 0.05$] weights and circulating testosterone concentrations [F(1,30) = 83.859; $P < 0.05$], but no significant main effect of time point or interaction between these two variables (Table 1). Similarly, in the chronic stress groups (Exp 2.1), there were significant main effects of age on body [F(1,30) = 2611.356; $P < 0.05$], testis [F(1,30) = 1876.009; $P < 0.05$], and adrenal [F(1,30) = 111.728; $P < 0.05$] weights and significantly lower circulating levels of testosterone [F(1,30) = 23.957; $P < 0.05$], but no significant main

TABLE 1. Mean (\pm SEM) body, paired testis, adrenal weights, and plasma testosterone concentrations in prepubertal (28 d old) and adult (77 d old) males in Exp 1

Measurement	Acute stress		Chronic stress	
	Prepubertal	Adult	Prepubertal	Adult
Body weight (g)	4.28 \pm 2.80	52.89 \pm 3.30 ^a	87.33 \pm 2.35	339.06 \pm 4.15 ^a
Testes (g)	0.70 \pm 0.03	3.15 \pm 0.05 ^a	0.66 \pm 0.03	3.14 \pm 0.04 ^a
Adrenal (mg)	27.00 \pm 2.00	65.00 \pm 3.00 ^a	28.00 \pm 1.00	65.00 \pm 3.00 ^a
Testosterone (ng/ml)	0.07 \pm 0.03	2.59 \pm 0.27 ^a	0.04 \pm 0.01	2.66 \pm 0.52 ^a

Prepubertal and adult males were exposed to a single 30-min session of restraint (acute stress) or a daily 30-min session of restraint for 1 wk (chronic stress). Note that data are collapsed across time points.

^a A significant difference between prepubertal and adult males; $P < 0.05$.

effect of time point or interaction between these two variables (Table 1). These peripheral indices of maturation indicate that the prepubertal males used in these experiments had not undergone any significant pubertal maturation and thus can be classified as prepubertal.

Hormonal measures.

Acute stress (Exp 1.1). In response to a single exposure to restraint, there were significant main effects of both age and time point on ACTH levels [$F(1,30) = 7.174$ and $F(2,30) = 21.654$, respectively; both $P < 0.05$], with no significant interaction. In particular, prepubertal males had higher ACTH levels than adults, whereas animals at both ages killed immediately after termination of the stressor had higher levels of ACTH compared with their baseline or recovery values (Fig. 1A). Furthermore, after a single session of restraint, two-way ANOVA revealed a significant interaction between age and time point on corticosterone secretion [$F(2,30) = 4.279$; $P < 0.05$]. In particular, prepubertal and adult males showed a significant elevation in corticosterone levels immediately after the stressor, but prepubertal males maintained a higher level of stress-induced corticosterone 45 min after termination of the stressor compared with the adults (Fig. 1B).

Chronic stress (Exp 2.1). Patterns of ACTH and corticosterone secretion changed dramatically in prepubertal and adult rats after chronic stress. Statistical analyses revealed a significant interaction of age and time point on both ACTH and corticosterone secretion [$F(2,30) = 32.241$ and 43.470 , respectively; both

$P < 0.05$] such that prepubertal males released more ACTH and corticosterone immediately upon termination of the stressor compared with adults, whereas 45 min after termination of the stressor, prepubertal animals showed lower ACTH and corticosterone levels than adults (Fig. 1, C and D).

CBG and free corticosterone concentrations: acute and chronic stress (Exp 1.1 and 2.1)

It is important to note that corticosterone values reported in Fig. 1, B and D, represent total plasma corticosterone concentrations, which reflect both free corticosterone and that bound to CBG. CBG is a high-affinity glycoprotein that binds to corticosterone, making it unavailable to target tissues such as the brain (34, 35). To establish whether free corticosterone concentrations paralleled total corticosterone values, we measured CBG and calculated free corticosterone in prepubertal and adult males exposed to acute and chronic stress. Prepubertal males had similar CBG levels compared with the adults in response to acute or chronic stress (Fig. 2, A and C). Thus, similar to the total corticosterone data presented in Fig. 1, B and D, prepubertal males experienced prolonged exposure to free corticosterone after an acute stress [$F(2,30) = 3.499$; $P < 0.05$; Fig. 2B], but after chronic stress, showed higher free corticosterone levels immediately after the stressor and significantly lower levels 45 min after stress compared with adults [$F(2,30) = 91.668$; $P < 0.05$; Fig. 2D].

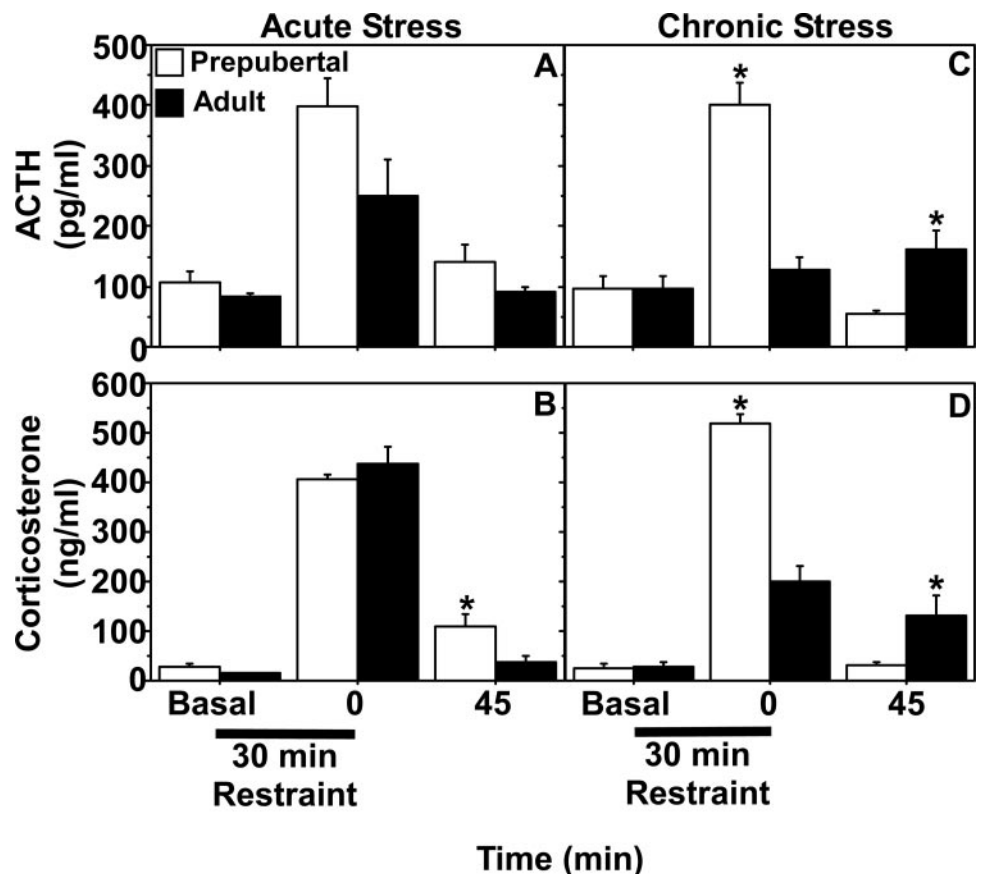


FIG. 1. Mean (\pm SEM) plasma ACTH (A and C) and corticosterone (B and D) concentrations in prepubertal and adult males exposed to a single 30-min session of restraint (acute stress; Exp 1.1) or a daily 30-min session of restraint for 1 wk (chronic stress; Exp 2.1). Asterisks indicate a significant difference between prepubertal and adult males.

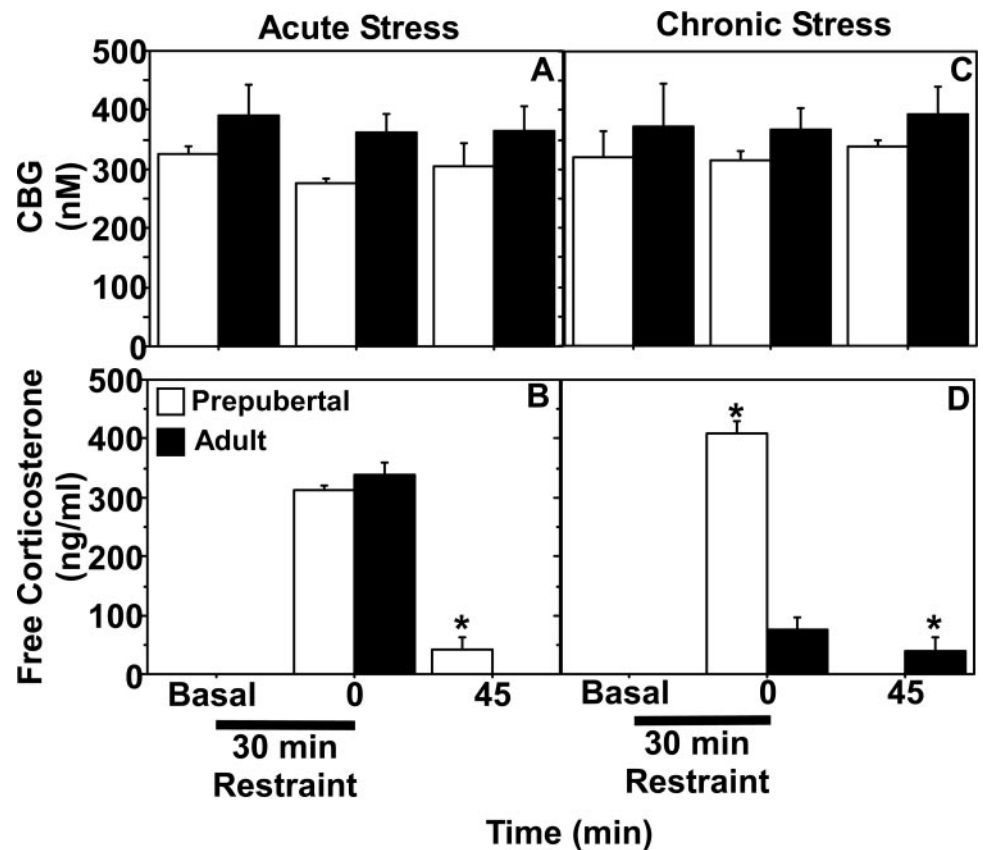


FIG. 2. Mean (\pm SEM) plasma CBG (A and C) and calculated free corticosterone (B and D) concentrations in prepubertal and adult males exposed to a single 30-min session of restraint (acute stress; Exp 1.1) or a daily 30-min session of restraint for 1 wk (chronic stress; Exp 2.1). Asterisks indicate a significant difference between prepubertal and adult males.

Experiential and developmental interactions on stress-induced neuronal activation and neuroendocrine plasticity

Peripheral measures. In the acute stress groups (Exp 1.2), there were significant main effects of age on body [F(1,30) = 5041.347; $P < 0.05$], testis [F(1,30) = 1298.03; $P < 0.05$], and adrenal [F(1,30) = 277.445; $P < 0.05$] weights, but no significant main effect of time point or interaction between these two variables (Table 2). Similarly, in the chronic stress groups (Exp 2.2), there were significant main effects of age on body [F(1,30) = 4714.578; $P < 0.05$], testis [F(1,30) = 1398.913; $P < 0.05$], and adrenal [F(1,30) = 46.418; $P < 0.05$] weights, but no significant main effect of time point or interaction between these two variables (Table 2). Thus, similar to the peripheral indices of maturation reported for Exp 1.1 and 2.1, these data indicate that the prepubertal males used in these experiments had not undergone any significant pubertal maturation and thus can be classified as prepubertal.

Stress-induced neuronal activation. To investigate whether the dramatically different stress responses to acute and chronic

stress before and after puberty were associated with differential neuronal activation, we employed FOS immunohistochemistry to assess neuronal activation (36) in various stress-responsive brain areas and regulatory nuclei of the HPA axis (32).

Acute stress (Exp 1.2). There were no significant differences in FOS-ir cell number in the mPFC at either age or time point (Fig. 3A). However, significant main effects of stress were found in the AcbSh [F(2,24) = 11.442; $P < 0.05$; Fig. 3B], vLS [F(2,24) = 35.996; $P < 0.05$; Fig. 3C], and CeA [F(2,24) = 7.366; $P < 0.05$; Fig. 3D], such that prepubertal and adult animals had higher FOS-ir cell numbers at both poststress time points (e.g. 0 and 45 min) compared with animals at the basal time point. A two-way ANOVA revealed a significant interaction of age and stress on FOS-ir cell number in the PVN [F(2,24) = 6.675; $P < 0.05$]. Specifically, compared with basal levels, there was an increase in FOS-ir cell number in the PVN immediately after termination of the stressor, but only in prepubertal males. However, 45 min after the stressor, both prepubertal and adult males showed

TABLE 2. Mean (\pm SEM) body, paired testis, and adrenal weights in prepubertal (28 d old) and adult (77 d old) males in Exp 2

Measurement	Acute stress		Chronic stress	
	Prepubertal	Adult	Prepubertal	Adult
Body weight (g)	89.61 \pm 2.46	355.29 \pm 2.80 ^a	86.06 \pm 1.76	347.12 \pm 3.33 ^a
Testes (g)	0.60 \pm 0.04	3.16 \pm 0.06 ^a	0.60 \pm 0.03	3.20 \pm 0.06 ^a
Adrenal (mg)	27.00 \pm 1.00	56.00 \pm 1.00 ^a	28.00 \pm 1.00	53.00 \pm 3.00 ^a

Prepubertal and adult males were exposed to a single 30-min session of restraint (acute stress) or a daily 30-min session of restraint for 1 wk (chronic stress). Note that data are collapsed across time points.

^a A significant difference between prepubertal and adult males; $P < 0.05$.

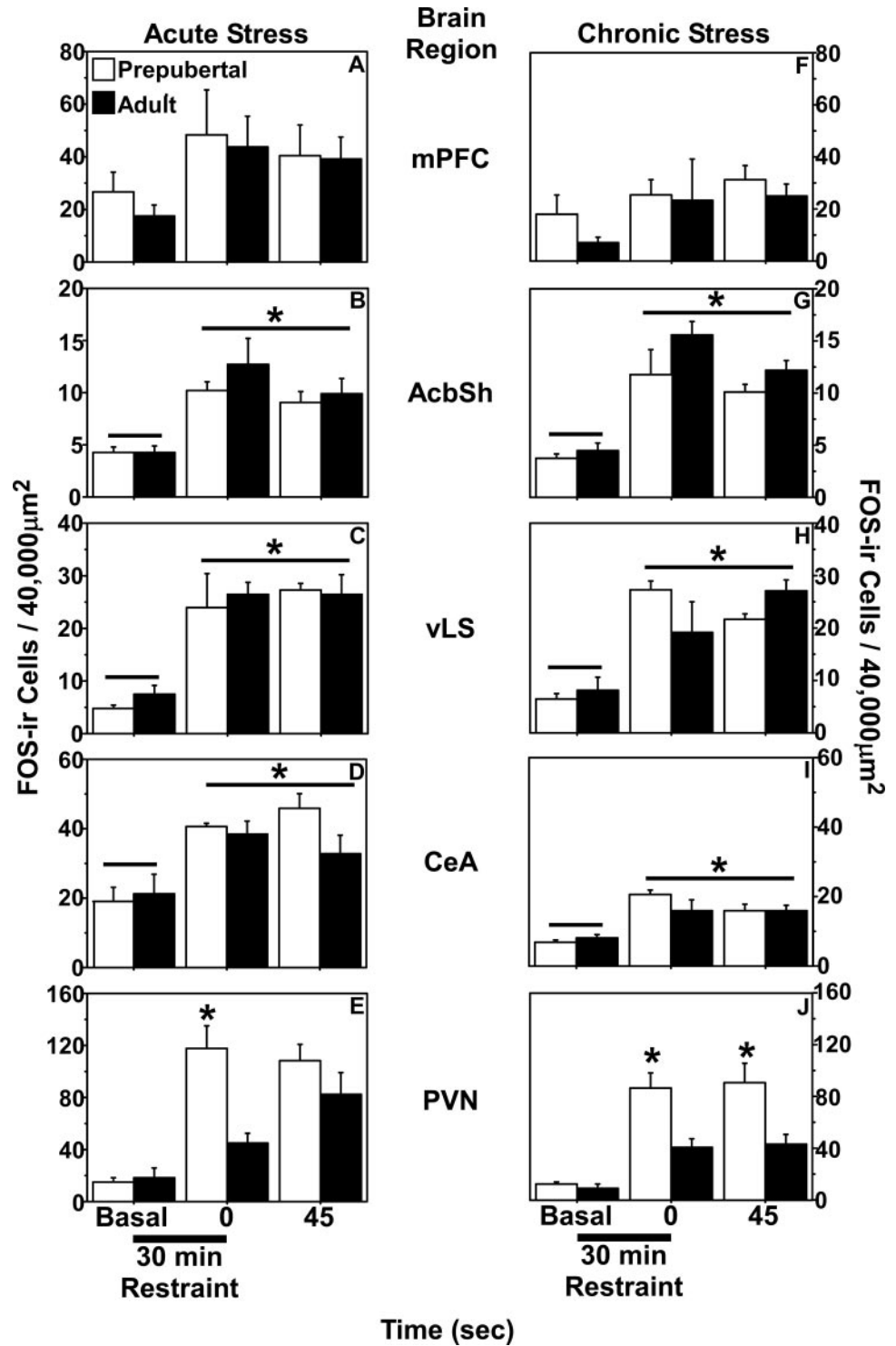


FIG. 3. Mean (\pm SEM) areal density of FOS-ir cell number per 40,000 μm^2 in mPFC (A and F), AcbSh (B and G), vLS (C and H), CeA (D and I), and PVN (E and J) of prepubertal and adult males exposed to a single 30-min session of restraint (acute stress; Exp 1.2) or a daily 30-min session of restraint for 1 wk (chronic stress; Exp 2.2). In B-I, *asterisks* indicate a significant main effect of stress; *asterisks* in E and J indicate a significant difference between prepubertal and adult males.

elevated FOS-ir cell numbers compared with the basal groups (Figs. 3E and 4, A–D).

Chronic stress (Exp 2.2). Similar to the acute stress group, there were no significant differences in FOS-ir cell number in the mPFC at either age or time point (Fig. 3F). Significant main effects of stress were found in the AcbSh [$F(2,24) = 35.207$; $P < 0.05$; Fig. 3G], vLS [$F(2,24) = 35.749$; $P < 0.05$; Fig. 3H], and CeA [$F(2,24) = 19.634$; $P < 0.05$; Fig. 3I], such that both prepubertal

and adult males had significantly higher FOS-ir cell numbers compared with animals at the basal time point. In the PVN, a significant interaction of age and time point on FOS-ir cell number was found [$F(2,24) = 3.797$; $P < 0.05$]. Compared with basal levels, stress increased PVN FOS-ir cell number in both prepubertal and adult males. However, at both poststress time points, prepubertal males had a higher number of FOS-ir cells compared with their adult counterparts (Fig. 3J and 4, G–L).

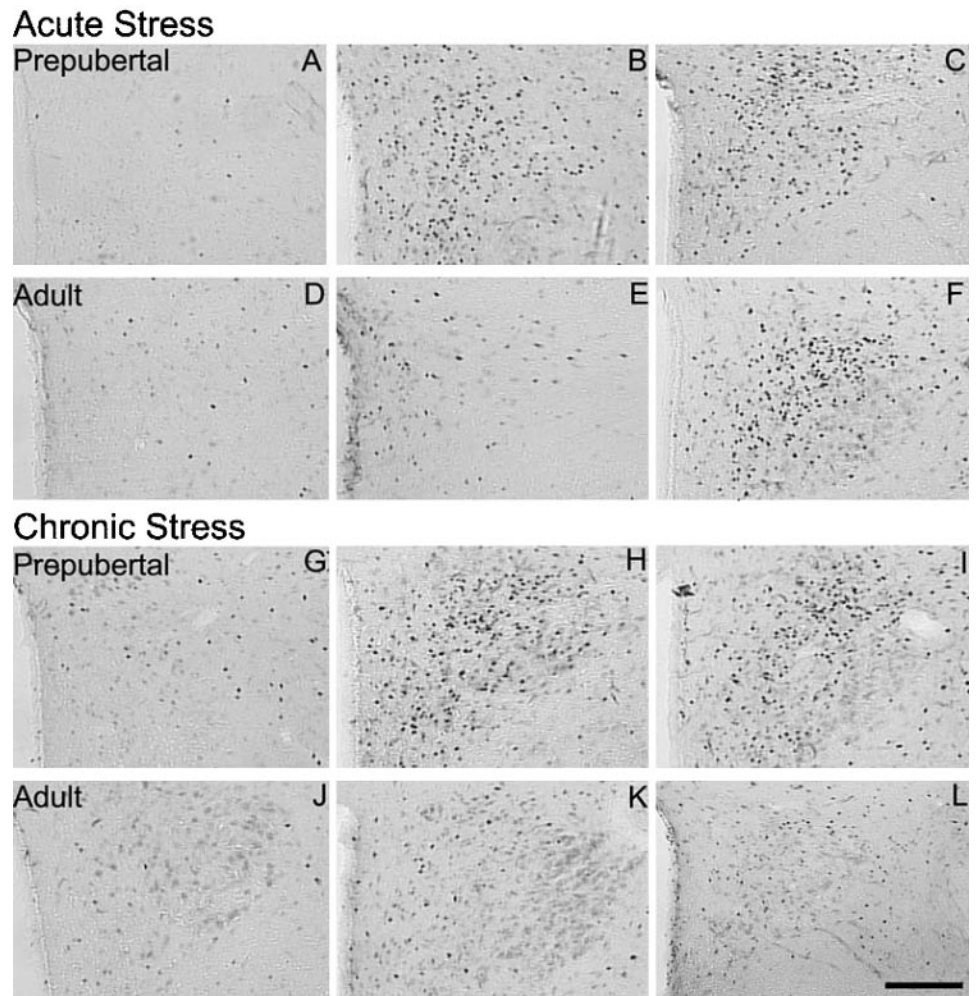


FIG. 4. Photomicrographs of FOS-ir cells in the PVN. *Upper panels*, Prepubertal (A–C; basal, 0 min, and 45 min, respectively) and adult (D–F; basal, 0 min, and 45 min, respectively) males exposed to a single 30-min session of restraint (acute stress; Exp 1.2); *lower panels*, prepubertal (G–I; basal, 0 min, and 45 min, respectively) and adult (J–L; basal, 0 min, and 45 min, respectively) males exposed to a daily 30-min session of restraint for 1 wk (chronic stress; Exp 2.2). Note the greater number of FOS-ir cells in the prepubertal PVN at the 0-min point after acute stress and at the 0- and 45-min points after chronic stress. Scale bar, 150 μ m.

Stress-induced activation of CRH vs. AVP cells in the PVN. The single-labeled FOS immunohistochemistry data revealed that the PVN is differentially activated depending on both pubertal development and experience with a stressor (Fig. 3, E and J, and Fig. 4). Thus, to investigate the cellular phenotype of the differentially activated cells in the PVN, we used triple-labeled fluorescence immunohistochemistry and confocal microscopy to quantify cell number and relative colocalization of FOS, CRH, and AVP. Figure 5 is a representative photomicrograph of the individual CRH, AVP, and FOS labels and the resulting merged triple-labeled image in the PVN of an acutely stressed prepubertal male 45 min after termination of the restraint session.

Acute stress (Exp 1.2). Similar to the single-labeled FOS results, we found a significant interaction of age and time point on FOS-ir cell number [$F(2,14) = 5.939$; $P < 0.05$; Table 1]. Specifically, compared with basal levels, stress increased PVN FOS-ir cell number immediately after termination of the stressor, but only in prepubertal males. However, 45 min after the stressor, both prepubertal and adult males showed elevated FOS-ir compared with the basal group (Table 3). There were no significant main effects or interactions of age and stress time point on the number of either CRH or AVP single-labeled cells in the PVN (Table 3).

There were few double-labeled AVP/FOS cells and triple-labeled FOS/CRH/AVP cells, and no statistically significant main effects or interactions were found regarding these measures (data not shown). There were also no statistically significant main effects or interaction of age and stress on the number of double-labeled CRH/AVP cells (data not shown). However, there was a significant interaction of age and stress on the percentage of CRH/FOS double-labeled cells in the PVN [$F(2,14) = 4.042$; $P < 0.05$]. Prepubertal males at the 0- and 45-min poststress points had a greater percentage of CRH/FOS-ir cells compared with their adult counterparts (Fig. 6A).

Chronic stress (Exp 2.2). Similar to our single-labeled FOS findings, two-way ANOVA revealed a significant interaction of age and time point on FOS-ir cell number [$F(2,14) = 3.691$; $P < 0.05$; Table 3]. Specifically, compared with basal levels, stress increased PVN FOS-ir cell number in both prepubertal and adult males. However, at both poststress time points, prepubertal males had higher FOS-ir cell numbers compared with their adult counterparts (Table 3). There were no significant main effects or interactions of age and stress time point on the number of either CRH or AVP single-labeled cells in the PVN (Table 3).

Similar to the acute stress groups, there were few double-

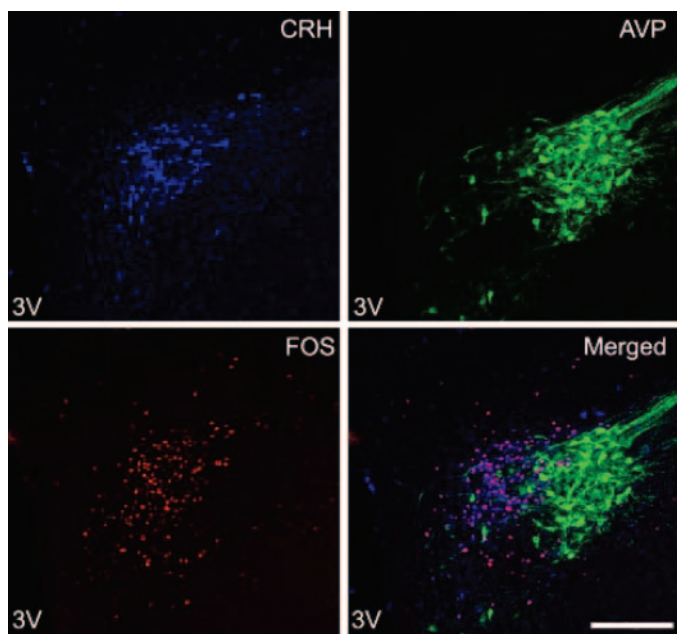


FIG. 5. A representative photomicrograph (stacked 1- μ m confocal sections) of the individual CRH (blue), AVP (green), and FOS (red) labels and the resulting merged triple-labeled image in the PVN of an acutely stressed prepubertal male 45 min after termination of the restraint session. Scale bar, 150 μ m.

labeled AVP/FOS cells and triple-labeled FOS/CRH/AVP cells, and no statistically significant main effects or interactions regarding these measures were found (data not shown). Furthermore, there were no statistically significant main effects or interaction of age and stress on the number of double-labeled CRH/AVP cells (data not shown). However, there was a significant interaction of age and time point on the percentage of CRH/FOS double-labeled cells in the PVN [$F(2,14) = 4.265$; $P < 0.05$]. Prepubertal males at the 0- and

TABLE 3. Mean (\pm SEM) number of single-labeled fluorescence FOS, CRH, and AVP cells in the PVN of prepubertal and adult males exposed to a single 30-min session of restraint (acute stress; Exp 1.2) or a daily 30-min session of restraint for 1 wk (chronic stress; Exp 2.2)

Label	Acute stress		Chronic stress	
	Prepubertal	Adult	Prepubertal	Adult
FOS				
Basal	41 \pm 2	13 \pm 7	25 \pm 7	34 \pm 16
0	193 \pm 7 ^a	83 \pm 10	218 \pm 28 ^a	104 \pm 29
45	223 \pm 20	137 \pm 36	317 \pm 61 ^a	107 \pm 28
CRH				
Basal	145 \pm 37	101 \pm 22	43 \pm 4	75 \pm 8
0	96 \pm 17	82 \pm 27	106 \pm 13	100 \pm 14
45	105 \pm 8	107 \pm 20	88 \pm 27	75 \pm 22
AVP				
Basal	132 \pm 14	196 \pm 13	100 \pm 14	135 \pm 10
0	153 \pm 17	188 \pm 34	143 \pm 17	227 \pm 32
45	176 \pm 7	170 \pm 24	162 \pm 25	140 \pm 29

Note that the FOS-ir cell numbers are higher in these analyses compared to the single-labeled areal density measures presented in Fig. 3, as former numbers represent the entire number of FOS-positive cells in the PVN, whereas the latter represent cells per unit area (*i.e.* FOS-ir cell/40,000 μ m²).

^a A significant difference between prepubertal and adult males within a time point; $P < 0.05$.

45-min poststress points had a significantly greater percentage of CRH/FOS-ir cells compared with their adult counterparts (Fig. 6B).

Discussion

These studies indicate that stress experience and pubertal development interact to modulate HPA axis reactivity and function. Specifically, after acute stress, prepubertal males exhibit a significantly prolonged stress response compared with adults. Interestingly, however, chronic stress results in a much shorter response in prepubertal compared with adult males. Additionally, we show that the differential stress reactivity in prepubertal and adult males is associated with differential neuronal activation in the PVN, such that in prepubertal animals, a larger proportion of CRH cells are activated in response to both acute and chronic stress compared with adults. These findings suggest that one neural locus of the experience-dependent plasticity exhibited by the HPA axis during pubertal development is the CRH-containing neurons in the PVN.

It is important to note that in addition to pubertal maturation, the experience of these animals may have been different. For example, factors such as the more recent weaning experience of the prepubertal animals or the potentially different social interactions experienced by both prepubertal and adult animals in their home cages before and/or after the stress session may have a differential influence on their stress reactivity. Future experiments will need to address how different social interactions and experience may influence stress responsiveness, and conversely, how differences in stress reactivity and experience may result in differential social interactions at these two profoundly different stages of development.

Dallman and colleagues (37) suggested that the reason for differential stress responsiveness in pubertal *vs.* adult animals is to protect ponderal growth in the juvenile and protect reproductive potential in the adult. In addition, we propose that the longer exposure to corticosterone after a single stressful experience in prepubertal animals may aid processes such as their memory formation and consolidation regarding the stressful event. Indeed, acute exposure to corticosterone had been shown to enhance memory formation and consolidation in adults (38). Furthermore, we posit that the faster termination of the stress response after chronic stress in prepubertal animals is to protect the maturing brain and other organs from the many detrimental effects of chronic corticosterone exposure. These hypotheses are currently under investigation.

Pubertal modulation of experience-dependent changes in hormonal stress responses

In the present study prepubertal males exposed to an acute stressor show a significantly longer corticosterone stress response compared with acutely stressed adults, in agreement with previous reports (12–14). Furthermore, this longer hormonal exposure prepubertally includes both total and free corticosterone. Interestingly, this pattern of hormone secretion was changed dramatically after chronic stress. Similar to other reports, adult males chronically exposed to the same

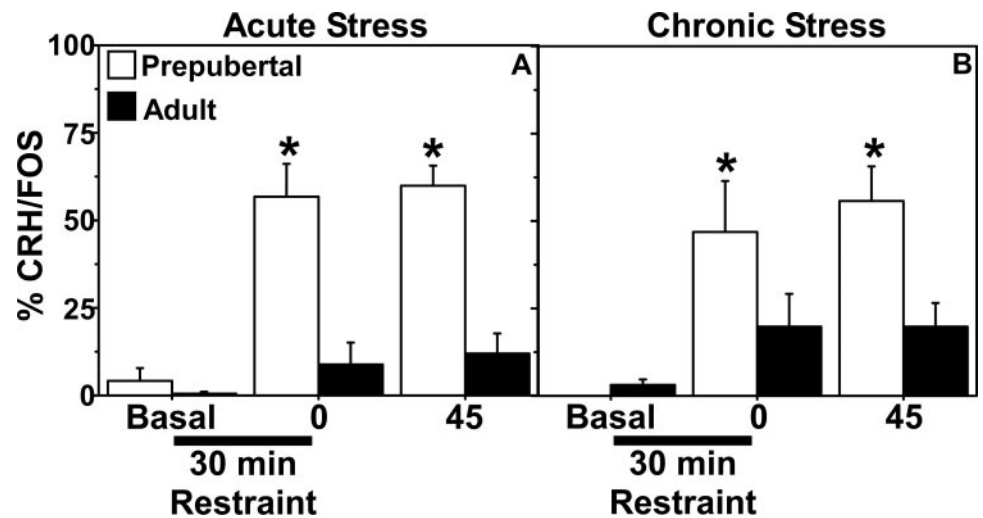


FIG. 6. Mean (\pm SEM) percent CRH and FOS double-labeled cells in the PVN of prepubertal and adult males exposed to a single 30-min session of restraint (acute stress; A; Exp 1.2) or a daily 30-min session of restraint for 1 wk (chronic stress; B; Exp 2.2). Asterisks indicate a significant difference between prepubertal and adult males.

stressors show a blunting of their ACTH and corticosterone stress responses (16–19). However, the chronically stressed prepubertal animals in the present study demonstrate two striking differences compared with adults: prepubertal males 1) show a significantly higher ACTH and total and free corticosterone response immediately after cessation of the stressor, and 2) have a return to baseline significantly faster upon recovery from the stressor. Taken together, the ACTH and corticosterone data indicate remarkable differences in stress reactivity depending on pubertal development and experience of the animal. It should be noted that a previous study showed that peripubertal males (40 d of age) react to 3 d of repeated restraint stress with a habituation of their ACTH response (37). Thus, a nonhabituating ACTH and corticosterone response to repeated stress may be specific to males in the early phases of pubertal maturation (*i.e.* 28 d of age) and suggests that experience-induced changes in stress responsiveness may also be dependent on the stage of pubertal development.

Corticosterone is a potent modulator of neuronal structure and function (39). For instance, corticosterone secretion induced by stressors alters dendritic branching (16, 40), dendritic spine density (41, 42), neurogenesis (43), and synaptic plasticity (44, 45) even after a single exposure to stress (42, 44). The prepubertal brain may be more sensitive than the adult brain to corticosterone, because an equivalent dose of corticosterone increased hippocampal *N*-methyl-*D*-aspartate receptor subunit expression (*e.g.* NR2A and NR2B) to a greater degree in prepubertal than adult males (46). Furthermore, brain regions that continue to mature during adolescence, such as the hypothalamus, hippocampus, amygdala, and prefrontal cortex (7–11), are also the most sensitive to corticosterone (2). Thus, it is likely that prepubertal and adult brains are differentially affected by the disparate exposure to corticosterone after acute and/or chronic stress. Whether the differential exposure to corticosterone in response to acute or chronic stress influences the present and/or future structure and function of the pubertal brain will require additional investigation.

Differential stress-induced neuronal activation before and after puberty

Stress activates many regions of the prepubertal and adult brain, including key regulatory nuclei of the HPA axis and various limbic and cortical areas (26, 32). Yet, despite dramatically different stress responses in prepubertal and adult males, many brain nuclei show similar activation after the stressor. For instance, acute and chronic stress lead to an increase in FOS-ir cell number in the AcbSh, vLS, and CeA, which is similar in prepubertal and adult animals, suggesting that these particular brain regions in prepubertal and adult animals are similarly activated by stress. It should be noted, however, that our analysis measured FOS as an index of neuronal activity, and even though FOS is a widely used marker of neuronal activation in response to restraint stress (21, 26–31), it is possible that other measures of neuronal activity would reveal differential stress-induced activation of the prepubertal and adult brain.

Although a number of similarities in FOS induction were observed at these two ages, a marked difference in stress-induced FOS was found in the prepubertal and adult PVN. In response to acute stress, we found the prepubertal PVN has a greater number of FOS-ir cells immediately after the 30-min session of restraint stress compared with adults, replicating a recent observation by Viau and colleagues (21). However, 45 min after termination of the acute stressor, the number of FOS-ir cells increases in the adult to reach levels similar to those found in prepubertal animals. After chronic stress, however, prepubertal animals exhibit a greater number of FOS-ir cells at both the 0- and 45-min points compared with adults, suggesting that differential activation of the PVN is dependent upon pubertal development and experience.

To probe differential activation in the context of PVN functional neuroanatomy, we triple-labeled FOS-, CRH-, and AVP-containing neurons before and after acute and chronic stress. Our data indicate that the differential activation between prepubertal and adult males is associated with differential activation of CRH-containing, but not AVP-containing, neurons. Specifically, prepubertal males exposed to

acute and chronic stress have a significantly higher percentage of CRH cells colocalized with FOS compared with adults at both the 0- and 45-min points.

Although a correlation between HPA activation (increased release of ACTH and corticosterone) and FOS expression in the PVN in the early phases of the stress response is well established (32, 47), it is difficult to correlate CRH cellular activation only with increased release of ACTH and corticosterone at later time points. Indeed, prepubertal males in the chronic stress condition show a relatively high number of CRH/FOS double-labeled cells in the PVN 45 min after termination of the stressor, when ACTH and corticosterone concentrations are relatively low. Similarly, adults exposed to an acute stressor have the highest number of FOS-ir cells 45 after termination of the stress, when ACTH and corticosterone levels return to baseline. Instead, these dissociations between CRH cellular activation and stress hormone secretion may represent cellular activity in a different context, such as replenishing CRH stores.

Regardless of the associations and dissociations between CRH/FOS-positive cells and hormonal responses, these triple-labeling data indicate two important points. First, because there were very few AVP/FOS cells in the magnocellular and parvocellular (the hypophysiotropic zone) regions of the PVN, it appears that the hormonal stress responses observed in our prepubertal and adult males are unrelated to activation of AVP cells in either subdivision of the PVN. It is possible, however, that with longer exposures to restraint, magnocellular AVP cells would have displayed higher levels of FOS immunostaining (48). Second, a large portion of the prepubertal and adult FOS-positive cells contain neither CRH nor AVP. The PVN is a heterogeneous nucleus composed of a diverse array of cell groups expressing various neurotransmitters and neuropeptides, including dopamine, enkephalins, TRH, substance P, and oxytocin (49). Thus, in addition to CRH, there are presumably other cell groups differentially activated in the prepubertal and adult PVN in response to acute and chronic stress, the physiological implications of which are presently unknown.

Conclusions

In conclusion, these data show that experience-dependent plasticity of the HPA axis is significantly modulated by pubertal development. Furthermore, these data are the first to show that the differential stress reactivity exhibited by prepubertal and adult males is associated with differential neuronal activation, particularly in the CRH-containing cells of the PVN. Whether these experience-dependent changes in neuroendocrine plasticity before puberty persist into adulthood and affect neurophysiological structure and function during pubertal development is currently being investigated.

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