Age- and Stress-Induced Changes in Corticotropin-Releasing Hormone mRNA Expression in the Paraventricular Nucleus of the Hypothalamus

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**Key Words**
Puberty · Adolescence · Hypothalamic-pituitary-adrenal axis · Restraint stress · Corticotropin-releasing hormone · Paraventricular nucleus

**Abstract**
In reaction to acute stress, prepubertal (25–28 days of age) animals demonstrate a prolonged adrenocorticotropic hormone (ACTH) and corticosterone response compared to adults (>65 days of age), while after chronic stress, prepubertal animals show a higher peak ACTH and corticosterone response, but a faster return to baseline compared to adults. Differential activation of corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN) of prepubertal and adult animals have been suggested to mediate these changes in stress responsiveness. The purpose of the present set of experiments was to further elucidate possible differences in PVN structure and function in prepubertal (28 days of age) and adult (77 days of age) male rats. The results indicate that PVN volume and somal size and cell number are similar in the parvocellular and magnocellular subdivision of the PVN before and after pubertal development. Furthermore, after a peripheral injection of the retrograde tracer Fluoro-Gold (FG), prepubertal and adult males demonstrate similar numbers of anterior pituitary projecting neurosecretory neurons in the parvocellular region of the PVN. Finally, using in situ hybridization we show that in response to acute stress, CRH mRNA in the PVN was affected by both age and stress such that prepubertal males have greater CRH expression than adults and both prepubertal and adult males show significant stress-induced increases in CRH mRNA. Interestingly, in response to repeated restraint, neither age nor stress significantly influence CRH expression. Together, these data indicate that both age and experience with stress interact to modulate CRH expression in the PVN.

**Introduction**
Although much is known about the function of the hypothalamic-pituitary-adrenal (HPA) axis during adulthood [1, 2], and its activity and regulation during neonatal development and old age [3–5], relatively little is known regarding the pubertal maturation of the HPA axis. In the few studies investigating the pubertal HPA axis, it has been consistently observed that although basal and stress-induced adrenocorticotropic hormone (ACTH) and corticosterone secretion are similar in prepubertal (25–28 days of age) and adult (>65 days of age)
animals, prepubertal animals have a significantly prolonged hormonal response compared to adults. Specifically, ACTH and total and free corticosterone levels of prepubertal male and female rats exposed to a single, acute physical and/or psychological stressor take at least 45–60 min longer to return to baseline compared to adults [6–11].

In addition to development, experience with stressors can also influence stress reactivity. For instance, in adults, repeated exposure to a homotypic stressor can lead to habituation of the hormonal stress response, such that stress hormone levels are blunted [12–15]. We have recently shown that, in contrast to the extended response observed after acute stress, repeated restraint stress (30 min/day for 7 days) resulted in prepubertal males exhibiting a higher peak ACTH and corticosterone (free and total) response immediately following the stressor, but a faster return to baseline compared to adults [10]. We have further demonstrated that this experience-dependent plasticity is associated with greater activation of corticotropin-releasing hormone (CRH), but not arginine vasopressin (AVP), neurons in the paraventricular nucleus of the hypothalamus (PVN) of prepubertal compared to adult males. Together, these results indicate that experience-dependent plasticity of the HPA neuroendocrine axis is significantly influenced by pubertal development, and that CRH containing neurons of the PVN are at least one neural substrate involved in modulating these changes [10].

As stress exposure during adolescence has been strongly correlated with the onset of depressive and anxiety disorders in adulthood [16–18], it is imperative to more fully understand the pubertal maturation of the neuroendocrine axis that controls stress responsiveness. In an effort to further delineate possible changes in the structure and function of the PVN in prepubertal and adult males, the purpose of the present set of experiments was 3-fold.

First, we characterized various morphological aspects (e.g. volume, somal size, neuron number) of the prepubertal and adult PVN. Second, we quantified the number of neurosecretory cells in the prepubertal (28 days of age) and adult (77 days of age) PVN. Experiment 1 was conducted to establish whether acute or repeated stress alters CRH mRNA expression differently in the PVN before and after pubertal maturation. Together, these results indicate that experience-related plasticity of the HPA neuroendocrine axis is significantly influenced by pubertal development, and that CRH containing neurons of the PVN are at least one neural substrate involved in modulating these changes [10].

Materials and Methods

Animals and Housing

For all experiments, male Sprague-Dawley rats were commercially obtained from Charles River (Harlan, N.Y., USA), housed 3 per cage in clear polycarbonate cages with wood chip bedding, and maintained on a 12-hour light-dark schedule (lights on at 07.00 h). Prepubertal and adult animals arrived on the same day, and prepubertal animals were weaned on the day of arrival (20 days of age), and evenly distributed among the experimental groups. Throughout the experiment, 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 NIH Guide for the Care and Use of Laboratory Animals and the Committee on Animal Research of The Rockefeller University.

Though the exact age span that encompasses adolescence is not precisely defined, the 3-week period between 30 and 50 days of age in the rat is associated with the greatest changes in somatic, behavioral, and neurobiological parameters from the juvenile to adult transition [19]. Animals in the present experiments were tested at either 28 or 77 days of age to investigate changes in PVN structure and function before and after puberty. More specifically, the animals tested at 28 days of age (prepubertal) were pass the neonatal and weanling stages of development (ending at approximately 21 days of age), but before the onset of puberty (approximately 30 days of age), while animals tested at 77 days of age were after pubertal maturation had occurred. Thus, any changes noted in the parameters investigated in the present study must be mediated by changes related to the pubertal transition into adulthood.

Experimental Design

Three experiments were conducted. Experiment 1 was conducted to obtain morphometric data on the prepubertal (28 days of age) and adult (77 days of age) PVN. Experiment 2 was performed to determine the number of parvocellular neurosecretory neurons in the prepubertal (28 days of age) and adult (77 days of age) PVN. Experiment 3 was conducted to establish whether acute or repeated stress alters CRH mRNA expression differently in the PVN before and after pubertal development. For all analyses, the experimenter was blind to the experimental condition of each subject.

Experiment 1

Prepubertal (28 days) or adult (77 days) animals were weighed and perfused after an overdose of sodium pentobarbital (100 mg/kg; n = 6). Animals were transcardially perfused with 100 ml of 0.9% heparinized saline followed by 150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were then post-fixed for 4 h in 4% paraformaldehyde and then cryoprotected in 30% sucrose at 4°C. Coronal brain sections were made on a cryostat (30 μm) and stored at –20°C in cryoprotectant until Nissl staining and morphometrical analyses were conducted (see below). A second group of prepubertal (28 days) and adult (77 days) animals (n = 5) were weighed, decapitated and the brains rapidly removed from the skull to obtain the wet weight of the brain (including cerebellum) at these two developmental stages.
Experiment 2
Prepubertal (23 days) and adult (72 days) animals were intraperitoneally injected with the retrograde tracer FG (25 mg/kg; Fluorochrome, LLC, Denver, Colo., USA; n = 4), to label neurosecretory neurons projecting to the anterior pituitary [20, 21], and 5 days later (i.e., at either 28 or 77 days of age) were transcardially perfused with 100 ml of 0.9% heparinized saline followed by 150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were then post-fixed for 4 h in 4% paraformaldehyde and then cryoprotected in 30% sucrose at 4°C. Coronal brain sections were made on a cryostat (30 μm) and stored at −20°C in cryoprotectant until immunohistochemistry was performed on the tissue (see below).

Experiment 3.1
Prepubertal (28 days) and adult (77 days) animals were weighed and rapidly decapitated by a 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 0), or 45 min after the stress session (n = 6 per age and time point). The plasma ACTH and free and total corticosterone responses of these animals have been published previously [10]. Animals were restrained in wire mesh restrainers in the prone position, 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). Brains were rapidly removed from the skull, snap frozen on powdered dry ice and stored at −70°C until sectioned on a cryostat (20 μm). Coronal sections were thaw-mounted on Fisher Brand Plus slides (Fisher Scientific, Pittsburgh, Pa., USA), dried, and stored at −70°C until processed for in situ hybridization (see below).

Experiment 3.2
Prepubertal (22 days) and adult (70 days) males were exposed to repeated restraint stress (30 min/day for 7 days) and brain tissue collected on the 7th day of stress (i.e. at either 28 or 77 days of age). The same stress procedure was used and the same time points measured as in experiment 3.1 (n = 6 per age and time point). The plasma ACTH and free and total corticosterone responses of these animals have been published previously [10].

Nissl Stain and Morphometrics. To estimate the volume of the PVN and somal size and areal density (cells per unit area) of neurons in both the magnocellular and parvocellular subdivisions of the PVN, every third coronal section throughout the rostral-caudal extent of the PVN (i.e. 90-μm intervals) was mounted on Fisher Brand Plus slides (Fisher Scientific, Pittsburgh, Pa., USA), stained with cresyl violet and coverslipped with DPX Mountant (Sigma, St. Louis, Mo., USA). For volumetric estimates and somal area measurements, PVN containing sections were analyzed using a computer-assisted morphometry system composed of a Nikon photomicroscope equipped with an Applied Scientific Instruments MS 2000XYZ computer-controlled motorized stage, a DAGE-MTI DC-330 video camera, a Gateway computer, and StereoInvestigator morphometry software (MicroBrightfield, Williston, Vt., USA). To measure volume, the boundaries of the PVN were outlined bilaterally on the computer screen under a 4× objective. The volume of each PVN was estimated by summing the PVN areas/1×10^6 × tissue depth analyzed, with tissue depth analyzed as: 30 μm × number sections analyzed.

For somal area, the boundaries of 30 somata from each the magnocellular and parvocellular subdivisions of the PVN were outlined on the computer screen under a 20× objective and area (μm²) was computed by the software (MicroBrightfield). For areal density (cell per unit area) of neurons in the magnocellular and parvocellular subdivisions of the PVN, an ocular grid was centered over the PVN at 4×, and then the magnification was increased to 40×. At least two, anatomically matched, bilateral counts were made for both the magnocellular and parvocellular areas of the PVN. All areal density data are expressed as mean number of cells/10,000 μm².

Fluoro-Gold Fluorescent Immunohistochemistry and Quantification
To better delineate the parvocellular neurosecretory neurons from the magnocellular neurosecretory neurons, tissue sections were double-labeled with AVP and FG, as the majority of AVP-containing cells are confined to the magnocellular field of the PVN [10]. For double labeling, a standard fluorescent immunohistochemical procedure was followed. Briefly, free-floating sections were rinsed 5 times for 10 min each in 0.1 M phosphate buffer (PB), blocked in 2% normal donkey serum (NDS) for 1 h in 0.1 M PB with 0.1% Triton X-100 (PBT), and then incubated in rabbit anti-FG (1:3,000; Fluorochrome, LLC) and guinea pig anti-AVP (1:10,000; Bachem; Torrance, Calif., USA) in 2% NDS in PBT for 48 h at 4°C. Sections were rinsed 5 times for 10 min each in 0.1 M PBT, then incubated in the appropriate donkey secondary conjugated to the CY-2 and CY-3 fluorescent chromogens (1:200; Jackson ImmunoResearch, West Grove, Pa., USA), and rinsed 3 times for 10 min each in 0.1 M PB. Sections were mounted on Fisher Brand Plus slides and coverslipped with Krystalon (EMD Chemicals; Gibbstown, N.J., USA).

FG and AVP staining in prepubertal and adult rats was examined by confocal microscopy using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, N.Y., USA) with a Zeiss LSM 510-META scanning confocal attachment. Sections were excited with an argon-krypton laser using the standard excitation wavelengths for CY-2 and CY-3. All tissue sections were excited using identical confocal and scanning settings. Stacked images were collected as 1-μm multitrack optical sections. To combine the two channels, LSM 3.95 software (Zeiss) running on a PC was used. A grid representing an area of 10,000 μm² was overlaid on the image. The AVP label was used as a marker of the magnocellular aspect of the PVN [10], and the areal density of FG-ir cells were counted in parvocellular portion of the PVN. At least two bilateral and anatomically matched sections of the PVN were examined in 1-μm steps. Cells were considered positively labeled when FG staining occurred in the same cell for at least three consecutive 1-μm scans. The average number of FG-positive parvocellular cells was computed for each animal, and then a group average was computed for each experimental group. Images were optimized for publication using Photoshop 7 (Adobe Systems, Mountain View, Calif., USA), and were adjusted only for brightness and contrast to enhance the signal-noise ratio.

In situ Hybridization and Densitometry
For production of the CRH riboprobe, a pluexscript SK vector containing a 1.2-kb fragment of the rat CRH cDNA was linearized...
with XhoI and SacI to make antisense and sense cRNA probes, respectively. Labeled RNA probes were synthesized by in vitro transcription of linearized, gel-purified DNA templates using the appropriate T3 and T7 polymerases with S35-labeled UTP. Full-length probes were separated from labeling reactions via size-exclusion columns before being mixed with hybridization buffer and measured for specific activity.

After specificity of the CRH antisense probe was demonstrated (fig. 1), a standard, previously published hybridization protocol was used with slight modifications [22]. Briefly, side-mounted sections were fixed in 3.7% formaldehyde for 10 min, then rinsed in a series of phosphate-buffered saline (PBS) washes, followed by rinses in triethanolamine-HCl (TEA) and TEA with acetic anhydride. Slides were then washed in 2× sodium chloride citrate (SCC) and dehydrated in a series of graded alcohols (70, 95 and 100%), delipidated in chloroform, and rinsed in 100% alcohol. For pre-hybridization, slides were exposed to hybridization solution (225 μl/slide), coverslipped, placed in a humidity chamber and incubated at 55°C for 1 h. Slides were then washed in 2× SCC and again dehydrated in a series of graded alcohols (70 and 95%). For hybridization, slides were exposed to hybridization buffer (225 μl/slide) with the 35S-labeled antisense or sense ribonucleotide probes (approximately 1× 106 cpm/slide), coverslipped, placed in a humidity chamber and incubated at 55°C overnight. Following hybridization, slides were washed in 2× SCC buffer and incubated with RNase A (10 μg/ml) in digestion buffer at 37°C for 30 min and then digestion buffer alone for 10 min. Slides were then rinsed in series of 2× SCC and 0.2× SCC washes at 55°C and dehydrated in a series of graded alcohols. Slides were air dried for 24 h and then apposed to Kodak BioMax MR film (Sigma) for 2 days to generate autoradiograms.

Relative optical densities (RODs) were measured from the autoradiograms using computerized image analysis software (MCID-M4, Imaging Research, Inc., St Catherines, Ont., Canada). Measurements were obtained for PVN from sections comparable to plate 24 of a standard rat atlas [23]. Background measurements were made from areas adjacent to the PVN and subtracted from the ROD. The same size circular tool, smaller than the size of the brain region being measured, was used to insure that the same size sample was measured from section to section and animal to animal (fig. 1). At least two bilateral measurements were made for each animal.

**Table 1.** Mean (± SEM) body weights and brain wet weights of prepubertal (28 days of age; n = 5) and adult (77 days of age; n = 5) male rats

<table>
<thead>
<tr>
<th>Measure</th>
<th>Prepubertal</th>
<th>Adult</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>96.00 ± 2.91</td>
<td>333.83 ± 9.52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Brain weight, g</td>
<td>1.43 ± 0.07</td>
<td>1.76 ± 0.08</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

For the three experiments, morphometrical and FG cell number data were analyzed using t tests, while CRH mRNA RODs were analyzed by using a two-way analysis of variance (ANOVA; age × time point). Significant main effects were analyzed with Tukey’s HSD tests and differences were considered significant when p < 0.05. All data are reported as means ± SEM.

**Results**

**Experiment 1**

There was a significant difference in both body weight and brain wet weight of prepubertal and adult males (t (8) = -23.900 and -2.908, respectively; table 1). For morphometrical analyses, prepubertal males had significantly lower body weights compared to adults (t (10) = -53.872; table 2). However, despite the significant differences in overall brain wet weight (table 1), the estimated volume of the prepubertal and adult PVN were similar (table 2). Furthermore, the somal area of magnocellular and parvocellular PVN neurons and areal density of neurons in the magnocellular and parvocellular regions of the PVN were similar in prepubertal and adult males (table 2).
Experiment 2

Five days after peripheral injection of FG, labeling was apparent in both the magnocellular and parvocellular regions of the PVN (fig. 2). There were no differences in the number of FG-positive cells per 10,000 µm² in the neurosecretory parvocellular region of the PVN in prepubertal and adult males (fig. 2). Furthermore, there were no differences in the number of FG-labeled cells in the magnocellular region of the PVN in prepubertal and adult males (data not shown).

Experiments 3.1 and 3.2

In response to acute stress, a two-way ANOVA revealed significant main effects of both age and stress on CRH mRNA expression in the PVN (F (1, 30) = 7.327 and F (2, 30) = 3.959, respectively, p < 0.05). Specifically, prepubertal males had significantly higher levels of CRH mRNA in the PVN compared to adults, independent of the stressor, while animals had higher CRH mRNA levels 45 min after termination of the stressor compared to basal levels, independent of age (fig. 3, left panel). Representative autoradiograms of these data are present in figure 4. In response to repeated restraint (30 min/day for 7 days), prepubertal and adult males had similar levels of CRH mRNA in the PVN, regardless of age and stress (fig. 3, right panel).

Discussion

These data indicate that despite robust differences in the stress-induced secretion of stress hormones in prepubertal and adult males, there are many similarities in the PVN of animals at these two developmental stages. First, the estimated volume of the PVN, as well as somal size and cell number, are similar in both the magnocellular and parvocellular regions of the prepubertal and adult PVN. Second, the number of neurosecretory cells, as indexed by uptake of peripherally injected FG in the anterior pituitary projecting parvocellular cells in the PVN,
are similar before and after pubertal development. Interestingly, in response to acute stress CRH mRNA in the PVN was affected by both age and stress; however, after repeated restraint neither age nor stress significantly modulated CRH expression.

Our morphometrical analyses revealed that independent of the overall difference in brain wet weight; the estimated volume of the PVN was similar in prepubertal and adult males. Furthermore, somal area and cell number in both the magnocellular and parvocellular aspects

**Fig. 3.** Mean (± SEM) relative optical density (ROD) of CRH mRNA in the PVN of prepubertal and adult males in response to acute restraint stress (30 min) or repeated restraint (30 min/day for 7 days). Significant main effects were found for both age and stress under the acute condition, while no significant differences were found in response to repeated restraint stress (n = 6).

**Fig. 4.** Representative autoradiograms of CRH mRNA in the PVN of prepubertal and adult males before (basal) or 45 min after a single 30-min session of restraint stress (acute stress group).
of the PVN were similar in prepubertal and adult animals. Somal size of neurosecretory cells, such as gonadotropin-releasing hormone cell bodies, have been shown to change in response to reproductive status [24]. However, it appears the neurosecretory cells in the PVN projecting to the anterior and posterior pituitary (parvocellular and magnocellular neurons, respectively) show comparable somal sizes before and after pubertal development. Though pubertal development dramatically regulates HPA responsiveness generally, and PVN activity specifically, it appears changes in gross morphology of the PVN at these two developmental ages does not contribute to these differences.

In addition to our cytoarchitectural study, we found no differences in the number of FG-labeled parvocellular (primarily CRH containing) neurons in prepubertal and adult animals. These data indicate that similar numbers of parvocellular PVN neurons project to the periphery before and after pubertal development. Thus, in parallel to our morphometrical data, it appears that the changes in HPA reactivity before and after adolescence are not mediated by differences in the number of neurosecretory cells in the parvocellular PVN.

In response to acute stress, both prepubertal and adult males show significant increases in CRH mRNA in the PVN. The stress-induced increase in CRH in adults is in agreement with previously published reports showing acute exposure to stress results in significant increases in CRH mRNA in the PVN [25–29]. The influence of acute stress on CRH expression in the prepubertal, postweaning PVN is less established. To date, only two studies to our knowledge have assessed stress-induced modulation of CRH mRNA in the prepubertal PVN [25, 30]. The study by McCormick et al. [30] showed significant stress-induced increases in CRH expression in the PVN of 30-day-old males, while a study by Viau et al. [25] reported a trend toward stress-induced increases in CRH mRNA in the PVN of 28-day-old males. Our present prepubertal data support these findings, but also suggest developmental decreases in CRH expression such that our prepubertal males had significantly higher CRH expression in the PVN compared to adults, regardless of stress.

In contrast to the response exhibited by prepubertal and adult males to acute stress, neither prepubertal nor adult males showed any significant changes in CRH expression in the PVN in response to repeated stress. In adults, exposure to a repeated homotypic stressor eliminates, or significantly reduces, the stress-induced increase in PVN CRH mRNA [26, 28], and thus our present data are in agreement with these previously published reports. Our data are the first report on how repeated stress affects CRH mRNA in the PVN of prepubertal animals. The present data indicate that modulation of CRH expression is similarly affected by repeated stressors before and after pubertal development.

Though changes in CRH expression in the PVN were noted in the present study, these changes do not parallel the significant pubertal differences in hormonal stress responsiveness. For instance, in reaction to acute stress, prepubertal males demonstrated a more prolonged ACTH and corticosterone response compared to adult, while after chronic stress, prepubertal animals show a higher ACTH and corticosterone response, but a faster return to baseline compared to adults [10]. Therefore, these data suggest additional factors contribute to the differential hormonal response demonstrated by prepubertal and adult animals in reaction to acute or repeated stress [6–11]. Future studies will need to clarify the role that possible differential release of CRH, sensitivity of the anterior pituitary to CRH and other secretagogues such AVP and/or the sensitivity of the adrenal cortex to ACTH may play in these differential responses.

In conclusion, we report that CRH expression in the PVN is modulated by both pubertal development and stress. However, the dramatic differential peripheral hormonal response induced by acute or repeated stress in prepubertal and adult males does not appear to be clearly associated with these changes in CRH mRNA. Furthermore, we show that the PVN cytoarchitecture is similar at these two developmental stages, and both have similar numbers of neurosecretory cells in the parvocellular region of the PVN. Together, these data argue that in addition to age- and stress-induced changes in CRH expression in the PVN, additional factors such as CRH release and/or sensitivity of the anterior pituitary to CRH may contribute to the robust hormonal differences exhibited by prepubertal and adult animals in response to either acute or repeated stress.

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Puberty and PVN

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