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Divergent stress-induced neuroendocrine and behavioral responses prior to puberty

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ABSTRACT

Following an acute stressor, pre-adolescent rats exhibit a protracted hormonal response compared to adults, while after repeated exposure to the same stressor (i.e., homotypic stress) prepubertal males fail to habituate like adults. Though the neurobehavioral implications of these changes are unknown, studying pubertal shifts in stress reactivity may help elucidate the mechanisms that underlie the increase in stress-related psychological and physiological disorders often observed during adolescence. Here, we investigated hormonal, behavioral, and neural responses of prepubertal (30d) and adult (77d) male rats before, during, or after acute stress (restraint), homotypic stress (repeated restraint) or heterotypic stress (repeated cold exposure followed by restraint). We found that prepubertal males exhibit prolonged corticosterone responses following acute and heterotypic stress, and higher adrenocorticotropic hormone and corticosterone responses after homotypic stress, compared to adults. Despite these significant age-dependent changes in hormonal responsiveness, we found that struggling behavior during restraint was similar at both ages, such that both prepubertal and adult animals exposed to homotypic stress struggled less than animals exposed to either acute or heterotypic stress. Across these different stress paradigms, we found greater neural activation, as indexed by FOS immunostaining, in the prepubertal compared to adult paraventricular nucleus of the hypothalamus, a nucleus integral for initiating the hormonal stress response. Interestingly, however, we did not find any influence of pubertal development on stress-induced activation of the posterior paraventricular thalamic nucleus, a brain region involved in experience-dependent changes in stress reactivity. Collectively, our data indicate that prepubertal and adult males display divergent hormonal, behavioral, and neural responses following a variety of stressful experiences, as well as a distinct dissociation between hormonal and behavioral reactivity in prepubertal males under homotypic conditions.

1. Introduction

Pubertal maturation is associated with many changes in neuroendocrine function, including significant shifts in the responsiveness of the hypothalamic–pituitary–adrenal (HPA) axis to stress [1–3]. In particular, though basal levels of adrenocorticotropin hormone (ACTH) and corticosterone are similar between prepubertal (25–30 days of age) and adult (>60 days of age) animals, prepubertal rats display greater and more protracted ACTH and corticosterone responses compared to adults following a diverse array of acute stressors, such as intermittent foot shock [4], ether vapor [5], and restraint [6–10]. Studies utilizing human subjects have also reported heightened hormonal stress reactivity during pubertal and adolescent development [11–13]. Though the physiological and neurobehavioural ramifications of these pubertal changes in stress responsiveness are largely unknown, the increases in stress-related physiological and psychological dysfunctions during puberty highlight the importance of examining factors that modulate stress reactivity during this crucial stage of maturation [14–16].

Experience with stressors is one potent factor that can shape an organism's stress responsiveness. For instance, adult animals repeatedly exposed to the same stressor (i.e., homotypic stress) typically show a reduced, habituated hormonal response compared to adults that experienced that stressor for the first time [10,17–24]. On the other hand, if an adult animal experiences homotypic stress and is then exposed to a novel stressor (i.e., heterotypic stress), it will exhibit an augmented, sensitized hormonal response compared to an animal exposed to that novel stressor alone [23,25,26]. Importantly, the ways in which experience modifies hormonal stress responsiveness are dependent on the pubertal development of the animal. Recent studies have shown that while adult animals display habituated ACTH and corticosterone responses following homotypic stress, prepubertal males show sensitized responses [10,24]. Though it is currently unknown if prepubertal and adult animals respond differentially to a heterotypic stress paradigm, these data clearly indicate that both age and experience interact to affect stress reactivity.

The neural substrates that mediate these age- and experience-dependent changes in stress reactivity are not fully understood. Previous reports have indicated that exposure to either acute or homotypic stress results in a greater level of neuronal activation, as indexed by FOS expression, in the prepubertal compared to adult paraventricular

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nucleus of the hypothalamus (PVN; [24,27]), particularly within the corticotropin-releasing hormone (CRH) cells [24]. Studies have also shown that shifts in the adult hormonal stress response following homotypic and heterotypic stressors are in part mediated by the posterior paraventricular thalamic nucleus (pPVT). More specifically, though lesions of the pPVT do not affect the hormonal response to an acute stressor [25], pPVT lesions lead to a lack of habituation after a homotypic stress [22], and greater sensitization following heterotypic stress [25]. The role of the pPVT in the unique experience-dependent plasticity of the hormonal stress response in prepubertal animals is currently unknown.

The present set of experiments examined the hormonal, behavioral and neural responses in prepubertal and adult animals exposed to acute, homotypic, or heterotypic stress. To investigate hormonal and neural reactivity, we exposed prepubertal and adult male rats to a single 30 min session of restraint stress (acute), 30 min of restraint stress for 8 consecutive days (homotypic), or 30 min of 4 °C cold stress for 7 consecutive days followed on the 8th day by a 30 min session of restraint stress (heterotypic) and measured plasma ACTH and corticosterone levels and FOS immunostaining in the PVN and pPVT. For behavior, we quantified struggling in our prepubertal and adult subjects during restraint, as a previous study in adults showed an association between hormonal responsiveness and struggling [28]. We hypothesized that the behavioral reactivity of the animals would reflect their hormonal responses, such that, for example under homotypic conditions, adults would show habituated hormonal and struggling responses [28], while prepubertal animals would show sensitized hormonal and struggling responses.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats arrived at 21 days age from a commercial supplier (Charles River Laboratories) and immediately housed 2 per cage in clear polycarbonate cages (45×25×20 cm) with wood chip bedding. Animals had at least 48 h to acclimate to the vivarium prior the initiation of any of the experiments detailed below. It is important to note that previous studies have shown similar pubertal-related changes in stress reactivity in response to acute and homotypic stress whether the animals were ordered from a breeder or bred in-house [10,24]. Moreover, we have found that basal corticosterone levels of 23 day old rats (i.e., the age our stress manipulations began under homotypic or heterotypic conditions) are not different in animals obtained from a commercial breeder or bred in our colony (unpublished observation). All animals had ad libitum access to food and water, and the animal room was maintained at 21±2 °C on a 12 h light–dark schedule (lights on at 0900 h). 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 approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University.

2.2. Experimental designs

Two experiments were conducted. Experiment 1 assessed hormonal stress reactivity and experiment 2 investigated both behavioral and neural reactivity in prepubertal and adult male rats exposed to acute, homotypic, or heterotypic stress. Specifically, in experiment 1, prepubertal and adult males were exposed to one of the three stress paradigms (i) acute stress (30 min of restraint stress), (ii) homotypic stress (30 min of restraint stress for 8 consecutive days), or (iii) heterotypic stress (30 min of cold room exposure at 4 °C for 7 consecutive days followed by 30 min of restraint stress on the 8th day). These stress paradigms were based on previously published studies investigating experience-dependent changes in adult stress reactivity [22,25,28]. Under the acute stress conditions, animals were exposed at 30 (prepubertal) or 77 (adult) days of age, while under homotypic and heterotypic conditions, animals began their stress exposures at 23 or 70 days of age and experienced their last restraint stress session at 30 or 77 days of age (Fig. 1). Though the specific age span that delineates pubertal development in the male rat is unclear, animals prior to 30 days of age are largely considered prepubertal, while animals after 70 days of age are considered reproductively mature adults [29,30]. Restraint stress was administered in a separate room from the colony by placing animals in the prone position in wire mesh restrainers, sized so that animals at these different developmental stages were equally restrained. Cold room exposure was administered in a separate room from the colony by placing animals in their home cages on cage racks in a 4 °C cold room.

On the final day of stress exposure (i.e., 30 or 77 days of age), all animals were weighed, rapidly decapitated, and a blood sample collected before (basal), immediately after the 30 min of restraint (Time 0), or 35 min following termination of restraint (n=6 per stress condition, age, and time point). Animals in the 35 min post-stress groups were returned to their home cages in the colony room until tissues were collected. All animals were killed between 1100 and 1300 h during their circadian nadir of ACTH and corticosterone release to minimize basal variations in these hormone levels [8]. Trunk blood samples were collected in Vacutainer K3 EDTA-coated tubes (Fisher Scientific, Pittsburgh, PA), spun down at 4 °C in a refrigerated centrifuge and plasma stored at −20 °C until radioimmunoassays were performed for ACTH and corticosterone (see below). Upon excision of the pituitary and adrenal glands, adipose tissue was removed and the glands were weighed to verify maturational stage as well as assess any age- and experience-dependent changes in these tissues based on body weight.

In experiment 2, prepubertal and adult males were exposed to acute, homotypic, or heterotypic stress exactly as described for experiment 1 (Fig. 1). However, to harvest tissue for immunohistochemistry, animals were perfused on the day of tissue collection between 1100 and 1300 h. Specifically, on the final day of stress exposure, animals were weighed and transcardially perfused before (basal), immediately after the stressor (Time 0), or 35 min following termination of the stressor (n=6 per stress condition, age, and time point). These specific time points were chosen for FOS analysis because of the differential stress-induced hormonal response exhibited at these times ([24] and experiment 1) as well as the significant, maximal increase in FOS immunoreactive cells observed in the PVN and pPVT between 15 and 60 min after the onset of a 30 min session of restraint stress in prepubertal and adult male rats [24,25,31]. Similar to experiment 1, animals in the 35 min post-stress groups were returned to their home cages in the colony room until tissues were collected. For perfusions, animals were given an overdose of sodium pentobarbital (150 mg/kg) and perfused with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were postfixed in 4% paraformaldehyde for 4 h and then stored in 20% sucrose at 4 °C until they were sectioned and processed for FOS immunohistochemistry (see below).

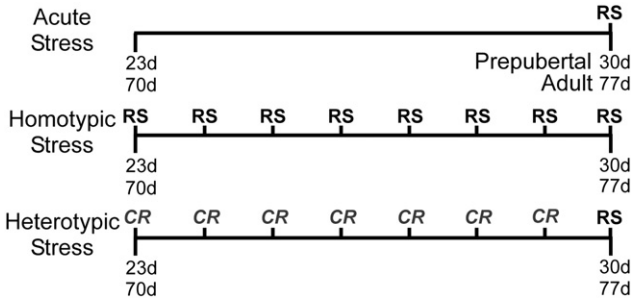

Fig. 1. Schematic of the experimental designs for experiments 1 and 2. Abbreviations: CR, cold room; d, days of age; RS, restraint stress.

2.3. Struggling behavior

The struggling behavior of animals in the restrainers under acute, homotypic, and heterotypic stress conditions in experiment 2 was examined. Specifically, digital camcorders (Canon DC210) were positioned above the area where the prepubertal and adult rats were restrained and their struggling behavior was recorded during their last restraint stress session. DVDs were made for later off-line examination. Struggling behavior was scored during the initial 15 min of restraint as prepubertal and adult animals engage in little struggling behavior after this time ([28], unpublished observation). Struggling behavior was operationally defined and quantified as any movement in which the animal rotated its head and neck more than a quarter turn or through the movement of its upper and lower body which caused the movement of the wire mesh restrainer. Each individual occurrence of struggling behavior was recorded, summed, and averaged within an age and stress condition to provide the mean frequency of struggling/15 min for the prepubertal and adult animals under acute, homotypic, or heterotypic conditions.

2.4. Sectioning, FOS immunohistochemistry, and cell counting

In experiment 2, 40 μm coronal brain sections were made on a cryostat (Jung Frigocut 2800 E) and stored in a cryoprotectant at −20 °C. Tissue sections were processed for FOS immunohistochemistry as described previously [32]. Briefly, free-floating sections were washed in 0.1 M PB and incubated for 10 min in 0.05% H_2O_2 in 0.1 M phosphate buffered saline (PBS). Sections were then washed in 0.1 M PB with 0.1% Triton X-100 (PBT), blocked in 2% normal goat serum (NGS) in PBT for 1 h, and incubated in anti-FOS (1:20,000; rabbit; Santa Cruz Biotechnology, Inc.) in 2% NGS in PBT for 24 h at 4 °C. Sections were then incubated in a goat anti-rabbit secondary antibody (1:200; Vector, Burlingame, CA) in PBT for 1 h and then incubated in avidin-biotin horseradish peroxidase complex (1:250; Vectastain ABC Kit, Vector) in PBT for 1 h, both at room temperature. Horseradish peroxidase was visualized with nickel-enhanced 3,3′diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 0.05% H_2O_2. Sections were washed, mounted on to Fisher Brand Plus slides (Fisher Scientific, Pittsburgh, PA), dried, dehydrated in increasing concentrations of alcohol, placed in xylenes, and coverslipped with DPX Mountant (Sigma-Aldrich, St. Louis, MO).

The areal densities (cells per unit area) of FOS-positive cell numbers were quantified in both the medial parvocellular (parv) and magnocellular (mag) regions of the PVN (Bregma −1.08 mm, [33]). The PVN was analyzed because previous reports indicated that the parv portion of the PVN displays differential neural activation in prepubertal and adult male rats following acute or homotypic stress paradigms [24,27]. FOS-positive cell counts were also made in the posterior paraventricular thalamic nucleus (pPVT; Bregma −3.24 mm; [33]) based on its role in modulating experience-dependent plasticity of the HPA axis [22,25].

Two brain sections containing each of these brain regions, separated by 120 μm and anatomically matched across subjects, were used for analysis. Cells were counted under a light microscope (Nikon, Eclipse E400) using an ocular grid. Ocular grid placement for these brain regions was based upon both a standard rat brain atlas [33] and a set of Nissl-stained sections adjacent to the sections processed for FOS. Each brain area was centered under a 10~ objective and then magnification increased to 40× so the grid covered an area of 15,625 μm^2. Two bilateral counts for each brain area were made and averaged. The data are presented as mean number of FOS-positive cells/mm^2.

2.5. Radioimmunoassays

ACTH and corticosterone radioimmunoassays were conducted using commercially available kits and performed as reported previously [9]. ACTH was measured using a ^{125}I kit (DiaSorin, Stillwater, MN) following the overnight incubation protocol (Option A), while corticosterone levels were obtained using Coat-A-Count kits (Siemens; Los Angeles, CA). For all assays, samples were run in duplicate and values were averaged. The intra-assay coefficient of variation (CV) and lower limit of detectability (LLD) for the ACTH assay were 14.4% and 9.98 pg/ml, respectively. For the corticosterone assay, the CV and LLD were 7.2% and 10.14 ng/ml, respectively.

2.6. Statistical analysis

All data are presented as the mean ± standard error of the mean (S.E.M.). Within each of the stress conditions, hormonal, somatic, and neural measures were analyzed using two-way (age × time point) analysis of variance (ANOVA). Struggling behavior was also analyzed by a two-way (stress condition × age) ANOVA. Significant main effects and interactions were further analyzed with Tukey's Honestly Significant Difference tests. Differences were considered significant when $P<0.05$. All statistical analyses were performed using GraphPad Prism software (version 5.04).

3. Results

3.1. Experiment 1

3.1.1. Somatic measures

Under all three stress conditions, there were significant main effects of age, but no main effects of time (i.e., basal, 0 min, 35 min), on body, pituitary, and adrenal weights. As expected, these weights were significantly less in prepubertal compared to adult rats (Table 1). Based on these somatic data, we can conclude that 30 and 77 day old animals are in different stages of pubertal and adult development. We also found that when we controlled for body weight, prepubertal animals under all three stress conditions had greater pituitary and adrenal weights compared to adults, such that the pituitary and adrenal glands represent a greater percentage of the animals overall body weight prior to pubertal development (Table 2). However, similar to gross wet weights, there were no main effects of time on these relative tissue weights.

Table 1
Mean (±SEM) body (g), pituitary (mg), and adrenal (mg) weights (experiment 1) and body weights (experiment 2) for prepubertal and adult males exposed to acute, homotypic, or heterotypic stress. Note that these data are collapsed across the stress time points (i.e., basal, 0 min, 35 min). Asterisks indicate a significant difference ($P<0.05$) between the ages within that stress condition. NA, not analyzed.

	Body weight (g)	Pituitary (mg)	Adrenal (mg)
Experiment 1			
Acute stress			
Prepubertal	110.8 ± 3.05	6.2 ± 0.00	28.8 ± 0.13
Adult	455.8 ± 1.71*	16.7 ± 0.01*	69.8 ± 0.43*
Homotypic stress			
Prepubertal	110.3 ± 3.53	6.5 ± 0.03	31.8 ± 0.12
Adult	404.0 ± 6.90*	14.5 ± 0.01*	72.6 ± 0.12*
Heterotypic stress			
Prepubertal	125.2 ± 1.70	6.8 ± 0.03	28.8 ± 0.06
Adult	417.0 ± 13.5*	16.0 ± 0.07*	66.3 ± 0.00*
Experiment 2			
Acute stress			
Prepubertal	107.8 ± 1.48	NA	NA
Adult	388.4 ± 9.71*	NA	NA
Homotypic stress			
Prepubertal	106.3 ± 1.87	NA	NA
Adult	394.4 ± 33.9*	NA	NA
Heterotypic stress			
Prepubertal	115.6 ± 2.46	NA	NA
Adult	419.0 ± 6.33*	NA	NA

Table 2
Mean (±SEM) percent pituitary and adrenal weights of body weight for prepubertal and adult males exposed to acute, homotypic, or heterotypic stress. Note that these data are collapsed across the stress time points (i.e., basal, 0 min, 35 min). Asterisks indicate a significant difference ($P<0.05$) between the ages within that stress condition.

Experiment 1	% Pituitary by body weight	% Adrenal by body weight
Acute stress		
Prepubertal	0.0056 ± 0.0001*	0.025 ± 0.008*
Adult	0.0037 ± 0.0001	0.016 ± 0.001
Homotypic stress		
Prepubertal	0.0056 ± 0.0002*	0.028 ± 0.003*
Adult	0.0036 ± 0.0001	0.018 ± 0.005
Heterotypic stress		
Prepubertal	0.0054 ± 0.0001*	0.023 ± 0.001*
Adult	0.0036 ± 0.0007	0.015 ± 0.001

3.1.2. Hormonal responses

In animals exposed to acute stress, we found a significant main effect of both age and time on plasma ACTH ($F(1,27)=4.69$ and $(2, 27)=90.25$, $P<0.05$, respectively) such that prepubertal males had higher levels of ACTH than adults, while both prepubertal and adult males had higher ACTH levels immediately following the stressor than animals at both ages either before or 35 min after the stressor had been terminated (Fig. 2A). For corticosterone levels, a significant interaction was found ($F(2,27)=20.30$, $P<0.05$) such that both prepubertal and adult animals displayed significant increases in corticosterone secretion immediately following restraint, but only the prepubertal animals continued to display a significant corticosterone response 35 min after the restraint was terminated (Fig. 2D).

In response to homotypic stress, we found a significant interaction of age and time on both ACTH and corticosterone levels ($F(2,27)=13.64$ and 23.74, $P<0.05$, respectively). Specifically, post-hoc tests revealed that while prepubertal and adult animals both showed significant increases in ACTH and corticosterone secretion at the time point immediately following termination of the stress, the rise of these hormones was significantly greater in prepubertal compared to adult males (Fig. 2B and E). However, both age groups had returned to baseline 35 min following the end of the stress session.

Following heterotypic stress, we found a significant main effect of time on the ACTH response ($F(2,27)=98.93$, $P<0.05$) such that both prepubertal and adult males showed elevated levels of ACTH immediately after the stressor, but animals at both ages returned to baseline 35 min after the stressor (Fig. 2C). In regard to corticosterone, a significant interaction was noted ($F(2,27)=3.07$, $P<0.05$). Here, prepubertal and adult animals both showed increases in plasma corticosterone levels immediately after the restraint was terminated, but the prepubertal animals continued to display a significantly higher corticosterone response 35 min following the end of the restraint session (Fig. 2F).

3.2. Experiment 2

3.2.1. Somatic measures

As in experiment 1, we found a significant main effect of age on body weight, with no main effect of time (Table 2). Again, these data indicate that these animals can indeed be classified as prepubertal and adult.

3.2.2. Struggling behavior

Two-way ANOVA revealed a significant main effect of stress condition on the struggling behavior of both the prepubertal and adult animals ($F(2,34)=5.42$, $P<0.05$). In particular, we found that independent of age, animals exposed to homotypic stress show significantly less struggling behavior than animals exposed to either acute or heterotypic stress (Fig. 3).

3.2.3. FOS response in PVNparv and PVNmag

Under acute stress conditions, there were significant interactions of age and stress on FOS immunoreactive cell number in both the PVNparv and PVNmag ($F(2,26)=2.93$ and 9.79, respectively, $P<0.05$). In the PVNparv, prepubertal males showed a significant increase in FOS cell number immediately and 35 min following termination of the stress, while adults only showed a significant increase at the 35 min post-stress time point (Fig. 4A). For the PVNmag, both prepubertal and adult males displayed significantly greater FOS responses following the stress, but the prepubertal animals had higher levels than the adults at both post-stress time points (Fig. 4D). The animals exposed to homotypic stress demonstrated significant main effects of both age and time such that prepubertal males had a greater number of FOS-positive cells than adults ($F(1,25)=8.90$, $P<0.05$), while animals immediately and

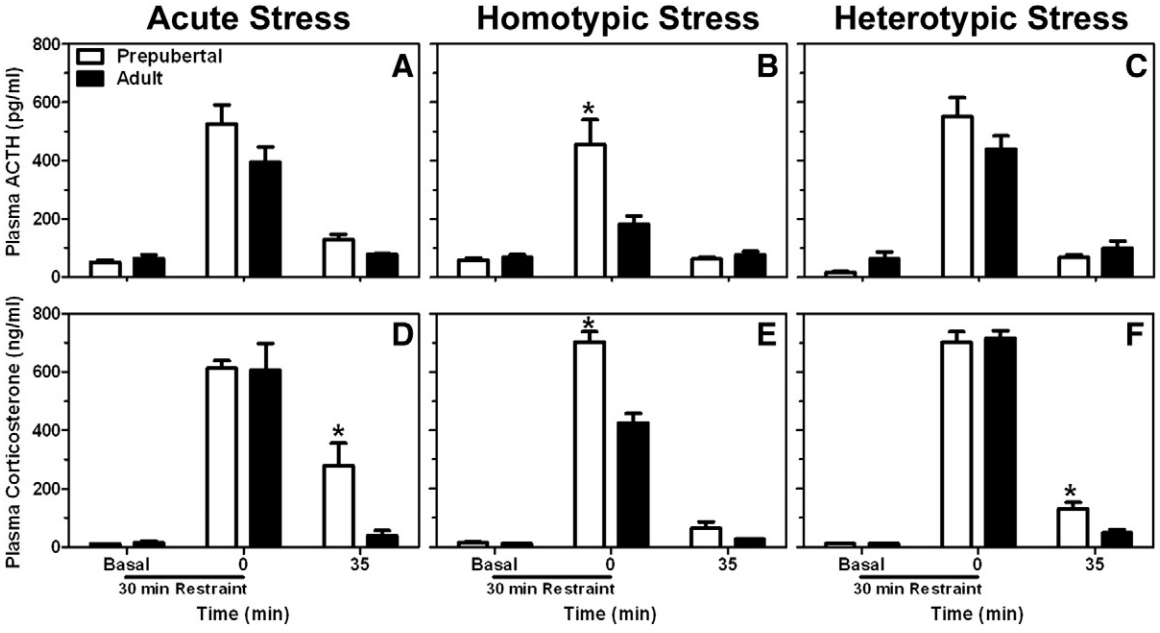

Fig. 2. Mean (±SEM) plasma ACTH (pg/ml) and corticosterone (ng/ml) in prepubertal (30 days of age) and adult (77 days of age) male rats exposed to acute stress (A and D), homotypic stress (B and E), or heterotypic stress (C and F). Asterisks indicate a significant difference between the ages at that time point.

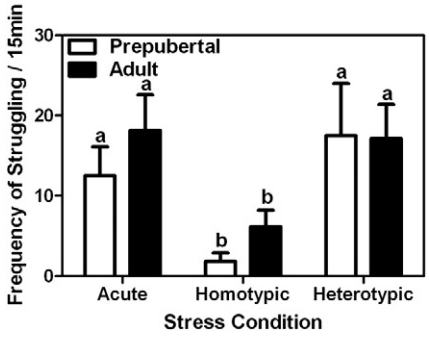

Fig. 3. Mean (±SEM) number of occurrences of struggling behavior in the initial 15 min of the last restraint session in prepubertal (30 days of age) and adult (77 days of age) male rats exposed to acute stress, homotypic stress, or heterotypic stress. Bars that have the same letter are not significantly different from one another.

35 min after termination of the stressor had significantly higher levels of neuronal activation compared to the animals before the stressor ($F(2, 25) = 7.99$, $P<0.05$; Fig. 4B and E). Similarly, animals exposed to heterotypic stress showed main effects of both age and time such that prepubertal animals had greater FOS-positive cell numbers than adults ($F(1,25) = 8.90$, $P<0.05$), and animals following the stressor had greater levels of FOS compared to the animals prior to the stressor ($F(2, 25) = 7.99$, $P<0.05$; Fig. 4C and F). Fig. 4G–L provide photomicrographs of the FOS response in both the parv and mag subdivision of the PVN in prepubertal and adult animals immediately following restraint (i.e., Time 0) under acute (Fig. 4G and J), homotypic (Fig. 4H and K), or heterotypic (Fig. 4I and L) stress conditions.

3.2.4. FOS response in pPVT

A two-way ANOVA revealed a significant main effect of time, but no effect of age or interaction between age and time, on FOS-positive cell number for the animals exposed to acute stress ($F(2,25) = 6.53$, $P<0.05$) such that animals at the post-stress time points had a significantly greater number of FOS cells in the pPVT compared to animals under basal conditions, independent of age (Fig. 5A). Though there were no significant effects of homotypic stress on FOS-positive cell number (Fig. 5B), heterotypic stress resulted in a main effect of time ($F(2,25) = 3.53$, $P<0.05$). Specifically, similar to the animals under acute stress conditions, both prepubertal and adult animals exposed to heterotypic stress showed significant increases in pPVT FOS-positive cell numbers after termination of the restraint (Fig. 5C).

4. Discussion

These data extend our basic understanding of the pubertal development of stress reactivity in many significant ways. First, under acute, homotypic, or heterotypic stress conditions, prepubertal males display differential hormonal responses compared to adults. Second, in contrast to the parallel association between behavioral and hormonal stress responsiveness in adults [28], we found a dissociation between struggling behavior and HPA reactivity in prepubertal animals exposed to homotypic stress. More specifically, although prepubertal animals display heightened ACTH and corticosterone responses compared to adults following homotypic stress, their struggling behavior shows an adult-like habituation pattern. Finally, we found greater PVN activation in prepubertal compared to adult animals under all three stress conditions, but no pubertal-related differences in stress-induced activation of the pPVT. These data emphasize the significant influence of pubertal development on stress reactivity and suggest divergent neuroendocrine and behavioral responses prior to puberty, particularly under conditions of homotypic stress.

Our hormonal data both compliment and extend the previous literature on age-related differences in stress reactivity. In particular, we found a protracted corticosterone response in prepubertal compared to adult males exposed to acute stress [4–6,8–10], as well as greater ACTH and corticosterone responses in prepubertal compared to adult males exposed to homotypic stress [10,24]. We also found that prepubertal and adult males exhibited a differential hormonal response to heterotypic stress, such that prepubertal males recovered to baseline more slowly after termination of the stressor than did adults. Thus, across these disparate stress paradigms, prepubertal males show either larger or more prolonged hormonal responses when compared to adults, suggesting that the HPA axis may be under greater drive and/or less negative feedback prior to puberty, though these possibilities have yet to be tested. It is important to note, however, that a recent study showed that adults display a significantly greater hormonal response compared to prepubertal males following an immune stressor (i.e., exposure to lipopolysaccharide) [32], indicating that age-related changes in hormonal reactivity are highly dependent upon both the type (psychogenic vs. systemic) and duration of the stressor.

Our hormonal data clearly indicate that age and experience interact to affect stress reactivity, but it is unknown what factor(s) may mediate this interaction. Pubertal-related changes in gonadal hormones levels, such as testosterone, may be involved as they are known to influence hormonal stress reactivity in adulthood [34,35] and increase dramatically throughout the pubertal stage of development [9]. Testosterone levels do not appear to affect the prolonged corticosterone response following acute restraint in prepubertal compared to adult male rats [6]. However, testosterone had been implicated in the changes in hormonal reactivity of mid-pubertal (i.e., 40 days of age) males after repeated restraint [36]. Thus, it is possible that pubertal changes in the hypothalamic–pituitary–gonadal axis may, in part, mediate these pubertal-dependent changes in HPA reactivity following homotypic and heterotypic stress.

In adult rats, hormonal responsiveness during restraint stress has been shown to parallel their behavioral reactivity, such that adult rats that have been repeatedly exposed to restraint (i.e., homotypic stress) show both a habituated corticosterone and behavioral struggling response compared to adults exposed to restraint for the first time (i.e., acute stress; [28]). Though we found congruent habituated hormonal and behavioral responses in adult males exposed to homotypic stress [28], as well as congruent responses in prepubertal and adult animals exposed to acute and heterotypic stress, we did not find this association between the hormonal and behavioral responses of prepubertal animals exposed to homotypic stress. More specifically, following homotypic stress, prepubertal animals displayed a habituated pattern of struggling behavior similar to that shown by the adults, but did not show any hormonal habituation as seen in the adults. It is presently unclear what mediates this inverse relationship between hormonal and behavioral responsiveness in prepubertal males under homotypic conditions, but is unlikely due to the hormonal differences per se as adrenalectomy does not affect restraint-induced struggling behavior, at least in adults [28]. However, our data do indicate that while some parameters of stress reactivity, such as hormonal responsiveness, are quite different between prepubertal and adult animals, other aspects of stress reactivity appear to be unaffected by pubertal development.

Though not compared directly, adult males failed to show the often observed sensitization under heterotypic compared to acute conditions in the context of both hormonal and behavioral responsiveness [25,28]. This is possibly due to differences between the experiments in the duration (30 min versus 4 h cold room exposure; [25]) and type (cold room versus swim stress; [28]) of the stressors used for heterotypic experience. Thus, it appears that not all heterotypic stress conditions lead to robust hormonal and behavioral sensitization in adults.

Using FOS immunoreactivity as a marker of neuronal activation, we found that the PVNparv and PVNmag showed greater levels of activation in prepubertal compared to adult animals following termination of acute stress. These PVNparv results are in accordance with two

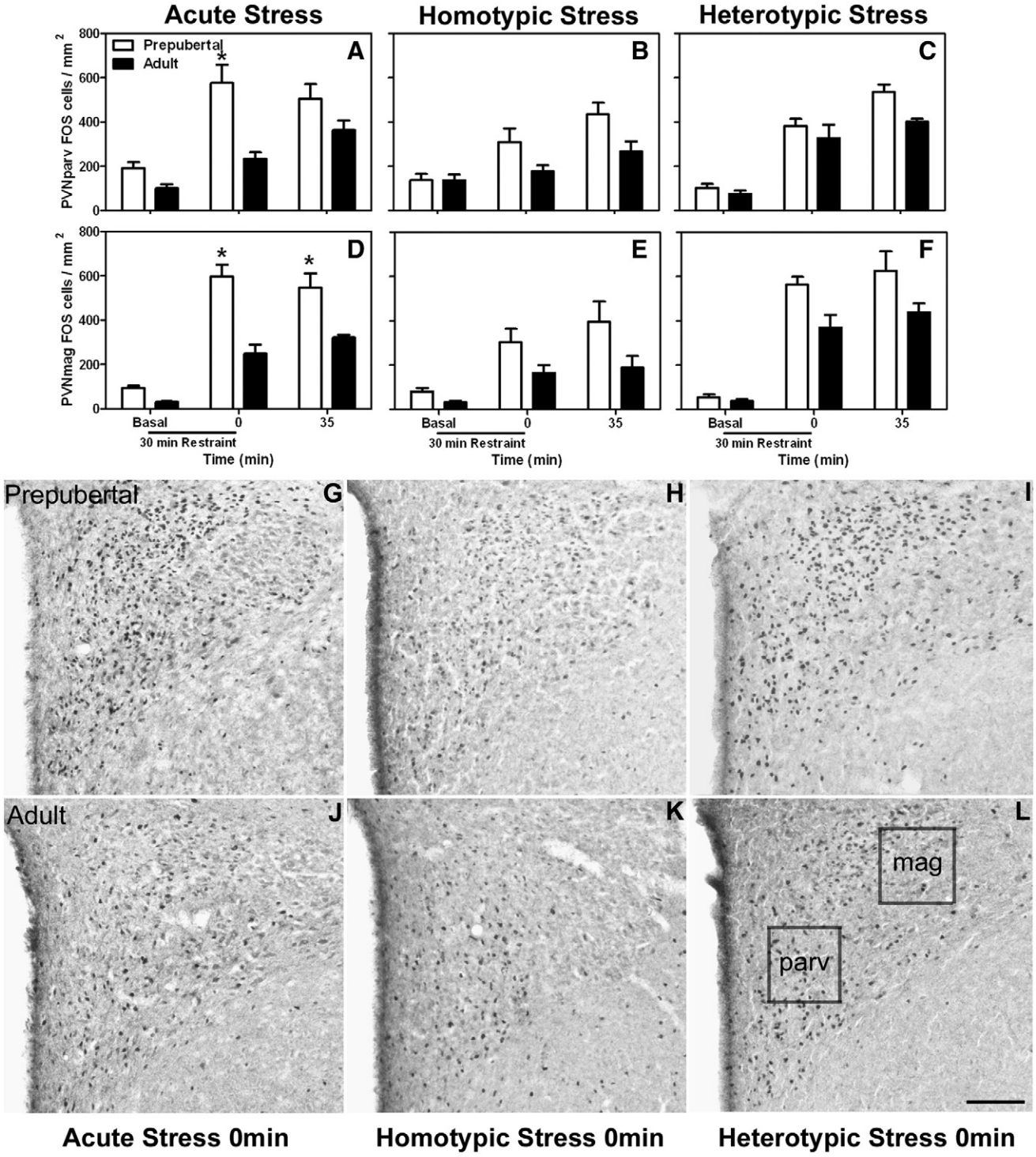

Fig. 4. Mean (±SEM) FOS-positive cells/mm^2 in the PVNparv (top data panels) and PVNmag (bottom data panels) in prepubertal (30 days of age) and adult (77 days of age) male rats exposed to acute stress (A and D), homotypic stress (B and E), or heterotypic stress (C and F). Asterisks indicate a significant difference between the ages at that time point. Representative photomicrographs of FOS-positive cells in the PVN of prepubertal and adult males immediately following the termination of the 30 min of restraint (i.e., Time 0) under acute (G and J), homotypic (H and K), or heterotypic (I and L) stress conditions. The squares in panel L indicate the approximate area and placement of the ocular grid used to quantify FOS-positive cell number in the parv (parvocellular) and mag (magnocellular) subdivisions of the PVN. Scale bar = 100 μm.

earlier studies that showed greater FOS responses in the prepubertal than in the adult PVN following acute and homotypic stress [24,27], and may in part contribute to the protracted hormonal response observed in prepubertal animals following these stressful experiences. It is important to note that differences in PVN activation between prepubertal and adult males cannot simply be accounted for by differences in cell density in, or volume of, the PVN as we have previously shown that these parameters are similar in the PVNparv and PVNmag before and after pubertal development [37]. The physiological implications of the greater FOS response we observed in the PVNmag prior to puberty are currently unclear. Since this area of the PVN is composed largely of the vasopressin and oxytocin neurons that project to the posterior pituitary [38], it is possible that, in addition to ACTH and corticosterone, prepubertal and adult animals show differential stress-induced hormonal vasopressin and/or oxytocin responses, a yet unexplored possibility.

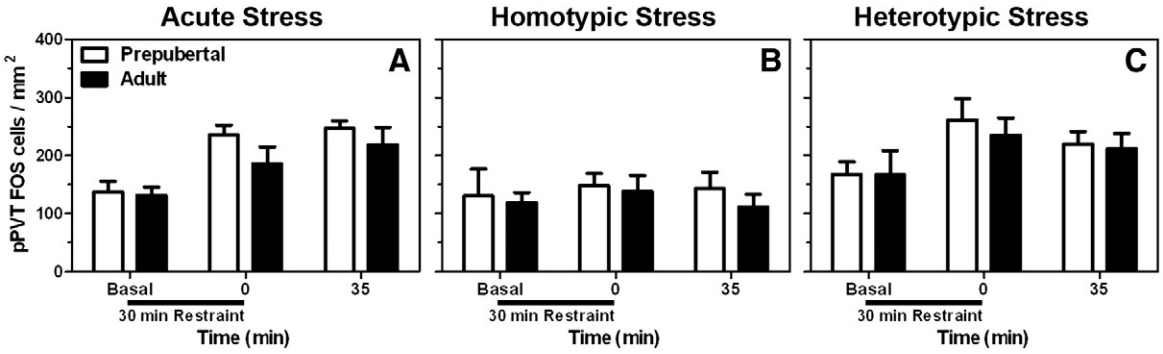

Fig. 5. Mean (±SEM) FOS-positive cells/mm^2 in the pPVT in prepubertal (30 days of age) and adult (77 days of age) male rats exposed to acute stress (A), homotypic stress (B), or heterotypic stress (C). Note that there were only significant main effects of time for animals under acute or heterotypic stress conditions, regardless of age.

Though we did not find any pubertal-related differences in the activation pattern of the pPVT in animals exposed to acute, homotypic, or heterotypic stress, this does not negate the possibility that the pPVT plays a role in these age- and experience-dependent changes in hormonal stress reactivity. Previous studies have established a crucial role of the pPVT in mediating the habituation and facilitated responses in reaction to homotypic and heterotypic stress, respectively, in adults [22,25]. Thus, future experiments will need to directly examine the role of the pPVT, perhaps via lesions studies, to elucidate its function in these unique experience-dependent hormonal changes exhibited by prepubertal animals. An important caveat regarding our FOS results, however, is that these data represent only general activation patterns within potentially heterogeneous brain areas. Thus, any age-related changes in hormonal stress reactivity may be due to the differential activation of cells of a particular phenotype, each with their unique afferents and efferents.

In addition to the role that differences in neural activation may play in these pubertal-related changes in hormonal responsiveness, it is possible that peripheral factors, such as differential rates of hormone synthesis in the pituitary and adrenal glands before and after puberty, may also contribute [9]. Along these lines, our somatic measures indicate that the relative wet weights of these glands are greater in prepubertal compared to adult animals. It is also possible that the sensitivity of the pituitary and/or adrenal glands to their respective secretagogues, such as corticotropin-releasing hormone, arginine vasopressin, or ACTH, may be differentially altered in prepubertal and adult animals after repeated exposure to stressors. Future studies will be needed to address these possibilities.

In conclusion, our data collectively indicate that pubertal developmental and stress experience interact to significantly modulate stress responsiveness. In response to an acute or heterotypic stressor, prepubertal males display a prolonged corticosterone response compared to adults, while following homotypic stress, prepubertal animals show greater ACTH and corticosterone responses than adults. Furthermore, though the adults showed congruent hormonal and behavioral responses following acute, homotypic, and heterotypic stress, we found a dissociation between these responses in prepubertal animals under homotypic conditions, such that their struggling behavior habituates following repeated exposure to restraint, while their hormonal responsiveness sensitizes. Our FOS analyses revealed both similarities and differences in activation patterns in the PVN and pPVT, regions integral in HPA function and experience-dependent plasticity. These brain areas clearly deserve further investigation to assess possible pubertal changes in their innervation and phenotypic composition that may in part mediate these shifts in responsiveness. Given the rather dramatic increase in stress-related psychological and physiological vulnerabilities during adolescence [12,29,39–44], it will be imperative to further explore these changes in stress reactivity and HPA function during the transition from puberty to adulthood.

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