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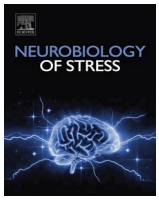

# Pubertal changes in the pituitary and adrenal glands of male and female rats: Relevance to stress reactivity

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ABSTRACT

The hormonal stress response mediated by the hypothalamic-pituitary-adrenal (HPA) axis changes significantly during puberty in a variety of species, including humans. For example, stress-induced adrenocorticotropic hormone (ACTH) and corticosterone responses are greater in prepubertal compared to adult rats, yet the mechanisms that mediate these age-related differences are unclear. It is possible that the pituitary and adrenal glands have higher hormonal concentrations prior to puberty, thus enabling a greater hormonal response if a stressor were to occur. Thus, we tested the hypothesis that resting levels of ACTH, and its precursor, proopiomelanocortin (POMC), are higher in the pituitary, and corticosterone levels are higher in the adrenals, of prepubertal compared to adult rats. Furthermore, to investigate any potential sex differences in these parameters, both males and females were assessed. Here we report that despite similar circulating plasma ACTH and corticosterone levels, prepubertal males and females have greater ACTH levels in the pituitary and greater corticosterone concentrations in the adrenals compared to adult males and females. Moreover, we show that POMC protein levels are significantly greater in the pituitary gland of prepubertal than adult rats, particularly in prepubertal females. These data suggest that increased glandular production of ACTH and corticosterone during puberty in part mediate pubertal differences in hormonal stress reactivity and highlight how each node of the HPA axis may contribute to these developmental changes. Given the dramatic increase in stress-related dysfunctions during puberty, continued study of all parts of the HPA axis will be imperative.

## 1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is the primary neuroendocrine axis responsible for mediating the hormonal stress response (Ulrich-Lai and Herman 2009). Specifically, upon experiencing a stressor, neurosecretory cells in the paraventricular nucleus (PVN) of the hypothalamus release corticotrophin-releasing hormone (CRH) and vasopressin (AVP) to stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH in turn stimulates the synthesis and secretion of glucocorticoids (i.e., primarily cortisol in primates, corticosterone in many rodent species) from the adrenal glands (Herman et al. 2003; Ulrich-Lai and Herman 2009). This hormonal response in part allows for physiological and behavioral changes to permit an individual to return to homeostasis following a stressor (Sapolsky et al. 2000).

Pubertal development is marked by significant changes in the function of the HPA axis (Green and McCormick 2016; Romeo 2018). For instance, though circulating basal ACTH and corticosterone levels are similar before and after pubertal development, prepubertal male and female rats show heightened and more prolonged stress-induced ACTH and corticosterone responses compared to adult males and females (Minhas et al. 2016). Though the physiological and neurobehavioral implications of these extended hormonal responses prior to puberty are unknown, these changes may contribute to the stress-related dysfunctions, such as mood disorders and obesity, often observed following a stressful adolescence (Turner and Lloyd 2004; Lee et al. 2014).

The mechanisms that mediate these developmental changes in HPA function are not entirely clear. However, it has been suggested that greater activation of the PVN (Romeo et al. 2006a; Lui et al. 2012; Baker et al. 2021) and/or less glucocorticoid negative feedback on the axis (Goldman et al. 1973) in prepubertal compared to adult animals may play a role in these age-related differences in HPA function. In addition to these neural mechanisms, peripheral factors might also contribute to the greater stress-induced hormonal responses observed prior to puberty. For instance, we have previously reported that the adrenal glands of prepubertal rats are more sensitive to ACTH than that of adults

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(Romeo et al. 2014). These adrenal data draw attention to the fact that the peripheral glands of the HPA axis may be contributing to developmental changes in hormonal stress reactivity.

Despite the greater stress-induced ACTH and corticosterone responses of prepubertal compared to adult rats (Minhas et al. 2016), little is known about how pubertal development affects the maturation of the pituitary and adrenal glands in the context of HPA function. Thus, the primary purpose of this study was to examine mediators at the level of the pituitary and adrenal gland that might be involved in developmental changes in stress reactivity. Furthermore, as sex differences have been noted in hormonal stress reactivity in adults, such that adult females often exhibit greater stress-induced hormonal responses than males (Handa et al. 1994; Armario et al. 1995; Rivier 1999; Heck and Handa 2019; Goel et al. 2022), a secondary purpose of this study was to explore potential sex differences in these parameters. To these ends, we used radioimmunoassays to examine ACTH and corticosterone concentrations in the pituitary and adrenal glands of prepubertal and adult male and female rats. In a follow up study we used western blots to assess protein levels of the precursor to ACTH, proopiomelanocortin (POMC), in the pituitary gland (Cawley et al. 2016). Based on greater hormonal stress reactivity observed prior to puberty (Green and McCormick 2016; Romeo 2018), we hypothesized that basal levels of ACTH and POMC in the pituitary and corticosterone in the adrenal glands would be higher in prepubertal compared to adult rats.

## 2. Materials and methods

### 2.1. Animals and housing

Male and female Sprague-Dawley rats obtained from our breeding colony at Barnard College were used for these experiments. The breeding stock was originally obtained from Charles River Laboratories (Wilmington, MA). On the day of birth, litters were culled to twelve pups, trying to maintain an even number of males and females. Litters were weaned at 21 days of age and housed in same-sex pairs. No more than two males and two females from a single litter were used to compose each experimental group. Before and after weaning, animals were housed in clear polycarbonate cages with bed-o' cobs ¼ inch bedding, had *ad libitum* access to food (Lab Diet #5012; PMI Nutrition International, LLC; Brentwood, MO) and water, and were maintained on a 12:12-h light/dark schedule (lights on at 0900h). Until the day of tissue collections, animals were left undisturbed throughout the experiment, expect for routine cage maintenance and care. 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 Animal Experimentation Guidelines from the Columbia University Institute of Comparative Medicine. All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University.

### 2.2. Experimental design and tissue collection

In the first experiment to assess plasma and glandular hormone concentrations, prepubertal (28 days of age) and adult (77 days of age) male and female rats were weighed and rapidly decapitated by a guillotine between 1100 and 1300h (n = 6 per age and sex). These times were chosen so that all animals were sacrificed at the same relative circadian time and to minimize circadian variations in HPA function (Romeo et al. 2006b). Trunk blood samples were taken in BD Vacutainer K3 EDTA-coated test tubes and spun in a refrigerated centrifuge at 850 rcf for 15 min at 4 °C. Plasma was immediately removed and stored at −20 °C until the radioimmunoassay (RIA) for ACTH and corticosterone were performed (see below). Pituitary and adrenal glands were also rapidly removed and snap frozen on dry ice, and stored at −80 °C until tissues were homogenized with a motor driven pestle in a buffer containing either 0.9% saline and 0.1 N HCl (pituitary samples) or 20% ethanol in 0.9% saline (adrenal samples), centrifuged at 4000 rcf, for 20 min, and supernatants diluted to 1:20 or 1:5 in their respective buffer. These samples were used to measure both pituitary ACTH content and protein concentrations and adrenal corticosterone content and protein concentrations. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL). ACTH and corticosterone concentrations (pg/ml and ng/ml, respectively) were then divided by the protein concentrations (mg/ml) and expressed as ACTH pg/mg protein or corticosterone ng/mg protein. Similar methods have been used previously to measure ACTH and corticosterone content in the pituitary and adrenal glands, respectively (Akana et al. 1992; Chisari et al. 1995; Ulrich-Lai and Engeland 2002; Watanobe and Yoneda 2003; Figueiredo et al. 2007; Foilb et al. 2011).

In a follow up experiment to assess POMC levels in the pituitary glands, prepubertal (30 days of age) and adult (70 days of age) male and female rats were weighed and rapidly decapitated by a guillotine during the same time of day as the first experiment (n = 6 per age and sex). Pituitary glands were rapidly removed and snap frozen on dry ice, and stored at −80 °C. Tissues were homogenized in a lysis buffer containing 1% SDS in $dH_2O$ with Roche complete, Mini, EDTA-free protease inhibitor cocktail (Roche, Diagnostics, Basal, Switzerland) and stored at −20 °C until western blotting (see below).

### 2.3. Radioimmunoassays

Radioimmunoassays for plasma and pituitary ACTH and plasma and adrenal corticosterone were conducted using commercially available kits (cat # 07–106102 and #07–120102, respectively; MP Biomedicals; Solon, OH) and performed as indicated by the supplier. For all assays, samples were run in duplicate and values were averaged. All duplicate samples had a coefficient of variation (CV) under 10%. The intra-assay CV and lower limit of detectability for the plasma ACTH and corticosterone assays were 3.4% and 5.5% and 5.17 pg/ml and 9.04 ng/ml, respectively, while for the pituitary ACTH and adrenal corticosterone assays the intra-assay CV and the lower limit of detectability were 5.9% and 3.6% and 6.51 pg/ml and 8.81 ng/ml, respectively.

### 2.4. Western blots

Protein concentrations from the supernatant were determined by the BCA protein assay (Pierce). Lysates (5 μg/lane) were subjected to SDS gel electrophoresis, blotted to a nitrocellulose membrane (Invitrogen; Carlsbad, Calif., USA) and probed with anti-POMC (1:5000; anti-rabbit Phoenix Pharmaceuticals, Inc, Burlingame, CA) and GAPDH as a loading control (1:5000; anti-mouse; Proteintech, Rosemont, IL). Using Kodak XAR film, bands were visualized by chemiluminescence (Pierce) according to manufacturer's instructions. Films were scanned and the relative optical densities (RODs) of immunoreactive bands were measured using open-access computerized image software (ImageJ). Once the background signal was subtracted from the film a rectangular tool was place over the immunoreactive POMC band and a ROD measurement was recorded. GAPDH bands were measured in an identical manner and used as a loading control to normalize potential differences in the amount of protein loaded in each lane. As the weights of POMC (~29 kDa) and GAPDH (~37 kDa) are relatively close, membranes probed for POMC were incubated in stripping buffer, containing 1% SDS and 0.7% β-mercaptoethanol, for 20 min at 50 °C, and then re-probed for GAPDH.

### 2.5. Statistical analyses

All somatic, hormonal, and protein measures were analyzed by two-way ANOVAs (age x sex). One adult female from the first experiment assessing plasma and glandular hormone concentrations was excluded from all statistical analyses due to an issue during tissue collection. Significant main effects and interactions were further analyzed with Tukey's honestly significant different tests. Differences were considered

significant when $p < 0.05$.

## 3. Results

The two-way ANOVA conducted on body weights showed a significant interaction between age and sex ($F(1,19) = 297.00$, $P < 0.05$), indicating that adults weighed significantly more than prepubertal animals and that adult males weighed significantly more than adult females (Fig. 1A). For gross overall weight of the pituitary, a two-way AVOVA revealed a significant main effect of age ($F(1,19) = 217.90$, $P < 0.05$), such that the adult pituitaries weighed significantly more than the prepubertal pituitaries, independent of sex (Fig. 1B). Despite this adult-biased difference in gross tissue weight, when expressed as percent body weight, there was a significant interaction of age and sex ($F(1,19) = 11.55$, $P < 0.05$) with adult males having the smallest pituitary by percent body weight than all other groups (Fig. 1D). In the context of overall protein concentration in the pituitary gland, a two-way ANOVA showed a significant main effect of age ($F(1,19) = 163.50$, $P < 0.05$). Specifically, adult pituitaries had significantly greater protein concentrations compared to the prepubertal pituitaries, independent of sex (Fig. 1F).

Similar to the pituitaries, a two-way AVOVA revealed a significant

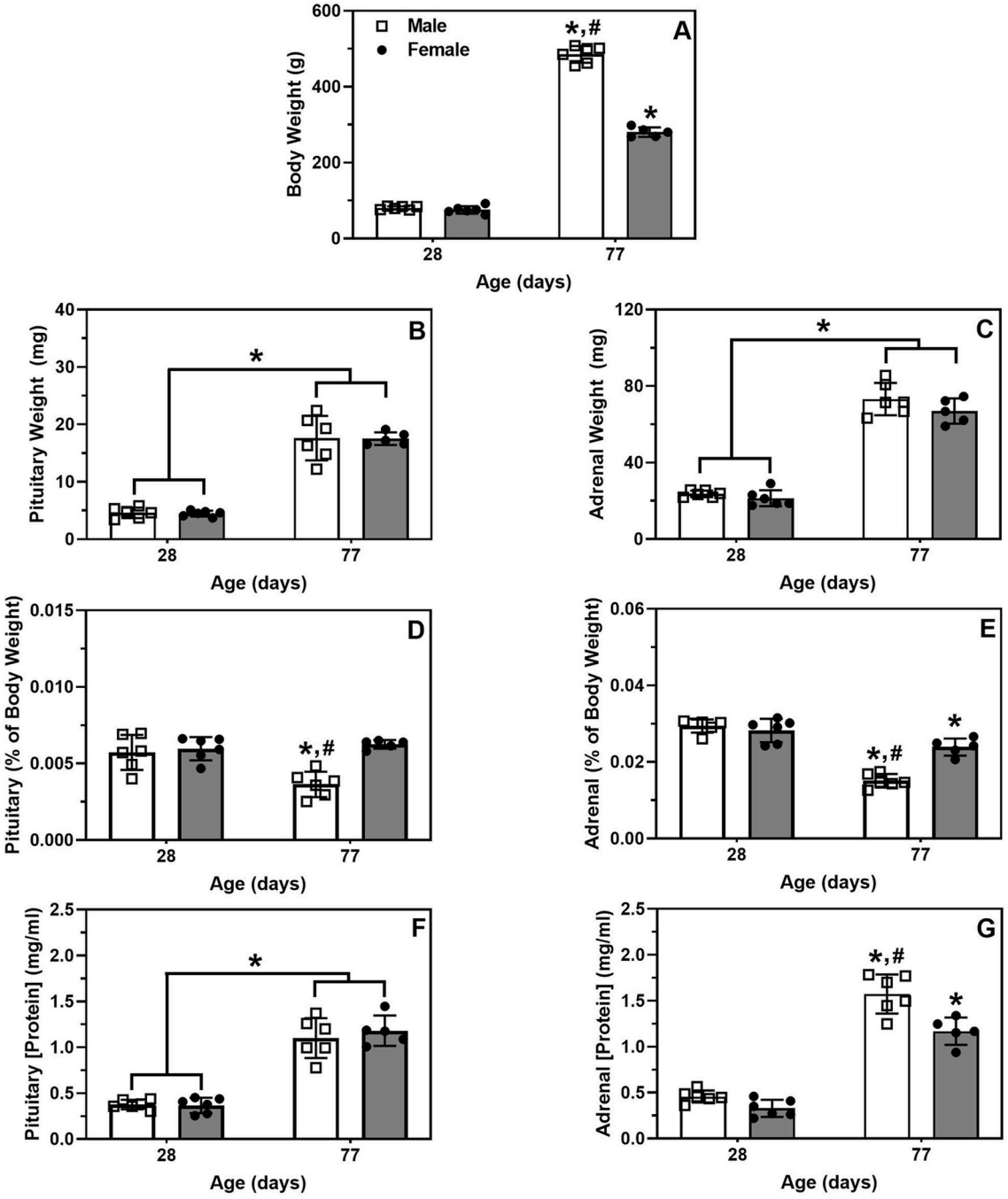


**Fig. 1.** Mean (+/− SEM) body weight (g; A), pituitary weight (mg; B), pituitary by % body weight (D), pituitary protein concentrations (mg/ml; F), adrenal weight (mg; C), adrenal by % body weight (E), and adrenal protein concentrations (mg/ml; G) in prepubertal (28 days of age) and adult (77 days of age) male (open squares) and female (closed circles) rats. In panels A, D, E and G, asterisks indicate that adults are significantly different from their prepubertal counterparts, while "#" indicates a significant difference between the sexes in adulthood. In panels B, C, and F, asterisks indicate a significant main effect of age.

main effect of age on the gross overall weight of the adrenal glands ($F(1,19) = 388.20$, $P < 0.05$), such that the adult adrenals weighed significantly more than the prepubertal adrenals, independent of the sex of the subject (Fig. 1C). Regardless of these differences in gross weight of the adrenals, when expressed by percent body weight, the ANOVA revealed a significant interaction between age and sex ($F(1,19) = 27.82$, $P < 0.05$). In particular, both prepubertal males and females had greater adrenal by percent body weight values than their adult counterparts and adult males had smaller adrenal by percent body weight compared to the adult females (Fig. 1E). Finally, in regards to the protein concentrations of the adrenal glands, a two-way ANOVA showed a significant interaction of age and sex ($F(1,19) = 5.51$, $P < 0.05$), indicating that adults had significantly greater adrenal protein concentrations than prepubertal animals and that adult males had significantly greater adrenal protein concentrations than adult females (Fig. 1G).

There were no differences in plasma ACTH levels between any of the groups, indicating similar circulating ACTH levels in prepubertal and adult males and females (Fig. 2A). However, the two-way ANOVA on ACTH concentrations in the pituitary gland revealed a significant main effect of age ($F(1,19) = 49.73$, $P < 0,05$), such that prepubertal animals had higher ACTH concentrations in the pituitary than adults, independent of sex (Fig. 2B). There was no significant main effect of sex or an interaction between age and sex on pituitary ACTH concentrations.

In a follow up study to assess POMC levels in the pituitary gland, the Western blot data showed a significant interaction between age and sex ($F(1, 20) = 5.56$, $P < 0.05$), such that POMC protein levels significantly decreased with age, but more so in females (Fig. 3A and B). These data suggest, that the elevated ACTH levels observed in the prepubertal compared to the adult pituitary gland may be in part due to increased POMC levels. Fig. 3B and C provide representative immunoreactive bands of POMC and GAPDH of prepubertal and adult males and females.

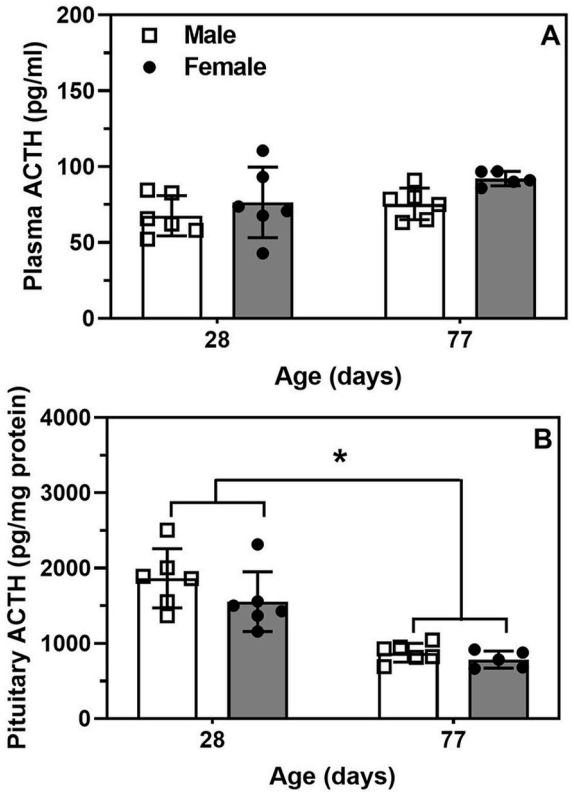


**Fig. 2.** Mean (+/− SEM) plasma ACTH (pg/ml; A) and pituitary ACTH concentrations (pg/mg protein, B) in prepubertal (28 days of age) and adult (77 days of age) male (open squares) and female (closed circles) rats. The asterisk in panel B indicates a significant main effect of age.

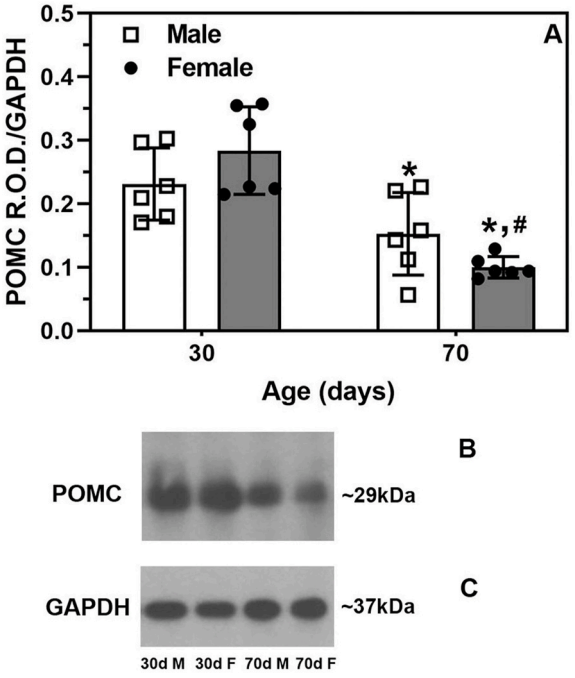


**Fig. 3.** Mean (+/− SEM) POMC ROD/GAPDH in prepubertal (30 days of age) and adult (70 days of age) male and female rats. In panel A, asterisks indicate that the adult males and females are significantly lower than their prepubertal counterparts, while # indicates a significant difference between the sex in adulthood. Panels B and C are representative bands of POMC (~29kDA) and GAPDH (~37 kDa) in prepubertal and adult males and females. Abbreviations, d, days of age; F, female; kDa, kilodalton; M, male.

Similar to ACTH levels in the plasma and pituitary, there were no differences in the circulating plasma corticosterone levels between any of the groups, indicating similar plasma corticosterone levels in prepubertal and adult males and females (Fig. 4A). However, the two-way ANOVA on corticosterone concentrations in the adrenal glands revealed a significant main effect of age ($F(1,19) = 30.30$, $P < 0,05$), such that prepubertal animals had higher corticosterone concentrations in the adrenal glands than adults, independent of sex (Fig. 4B). There was no significant main effect of sex or an interaction between age and sex on adrenal corticosterone concentrations.

## 4. Discussion

We report here that despite similar circulating basal levels of ACTH and corticosterone and greater overall protein concentrations in the adult pituitary and adrenal glands, the prepubertal pituitary has greater ACTH and POMC concentrations and prepubertal adrenal has greater corticosterone concentrations than adults. These data suggest that the changes in the hormonal content of the pituitary and adrenal glands prior to puberty might contribute to the changes in HPA function observed during this stage of maturation.

In the context of the pituitary, as both the POMC and ACTH levels were higher in the prepubertal rats, these data suggest increased production of POMC prior to puberty is driving the elevated ACTH levels, with little pubertal change in the activity of PC1/3, a convertase that cleaves POMC into ACTH (Cawley et al. 2016). Regardless of specific association between POMC and its cleavage to ACTH, it would appear the greater ACTH concentrations in the pituitary prior to puberty would permit the prepubertal pituitary to mount a great and/or more sustained ACTH response following a stressor, as has been previously demonstrated (reviewed in; (Green and McCormick 2016; Romeo 2018).

The mechanisms that might drive greater POMC expression in corticotropes prior to puberty are unknown. Steroid hormone receptors,

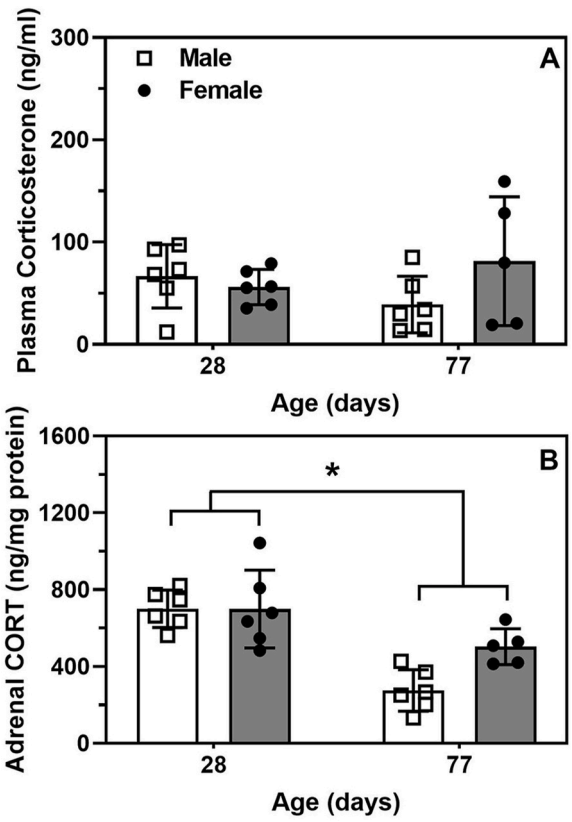


**Fig. 4.** Mean (+/− SEM) plasma corticosterone (ng/ml; A) and adrenal corticosterone concentrations (ng/mg protein, B) in prepubertal (28 days of age) and adult (77 days of age) male (open squares) and female (closed circles) rats. The asterisk in panel B indicates a significant main effect of age.

including androgen and estrogen receptor α, have been found in corticotropes (Mitchner et al. 1998; Maejima et al. 2009). Thus, it is possible that the significant increase in gonadal hormone secretion that occurs during pubertal maturation (Pignatelli et al. 2006) might play a role in regulating changes in POMC activity during this stage of development. It would be interesting to test whether the administration of adult-like levels of gonadal hormones, such as testosterone and estradiol, to prepubertal males and females would lead to decreases in POMC and ACTH concentrations in the pituitary. However, given the variety of transcription factors able to regulate POMC expression in corticotropes, such as NeuroD1 and NF-κB (Jenks 2009), it is unlikely that changes in gonadal hormones would be the sole mediator of the pubertal decrease in the levels of POMC and ACTH.

It is currently unclear what mechanisms mediate the greater corticosterone concentrations in the prepubertal compared to adult adrenal gland. However, our data would suggest greater levels of various factors along the steroid synthesis pathway, such as StAR, CYP11A, 3β-HSD, and CYP11β1, in the prepubertal compared to adult adrenal gland. Future studies measuring the expression of these factors before and after pubertal development would help clarify this possibility. Independent of the particular mechanism that regulates these age-related changes, it would appear the greater corticosterone concentrations in the adrenal gland prior to puberty would allow for the greater adrenal corticosterone response often observed following a stressor in prepubertal compared to adult animals (reviewed in; (Green and McCormick 2016; Romeo 2018).

We found no differences between males and females in the context of ACTH or corticosterone concentrations in the pituitary or adrenal glands, respectively. However, we did find sex differences in the adults in the context of the percent body weight of the pituitary and adrenal glands, such that adult females had greater pituitary and adrenal by percent body weights than their male counterparts. These gross measures of the pituitary and adrenal gland align with the known sex difference in hormonal stress reactivity in adult male and female rats, with some reports indicating that female rats exhibit greater ACTH and corticosterone responses (Handa et al. 1994; Armario et al. 1995; Rivier 1999; Heck and Handa 2019; Goel et al. 2022). It should be noted, however, that these gross measures are based on the entire pituitary and adrenal gland, and not just the anterior lobe or adrenal cortex. Thus, additional histological experiments would be required to better understanding whether these differences are due to changes within specific regions or zones of these glands.

We did find a subtle sex difference in POMC levels in adults. Specifically, we found that adult females had lower POMC levels than adult males. Given the similar levels of ACTH we observed in adult males and females, these data suggest that adult females may have greater levels of PC1/3, and/or that adult males have lower levels of PC1/3, ultimately resulting in these similar ACTH levels. It should be noted, we attempted to quantify PC1/3 levels via Western blot, but we were not able to get specific and reliable staining with several of the commercially available antibody we tested (unpublished observation). Perhaps future studies could quantify mRNA levels using alternative methods, such as RT-qPCR, to test this possibility of different PC1/3 expression in adult males and females.

Given the increased glandular ACTH and corticosterone concentrations in the prepubertal males and females compared to the adults, these data suggest the greater stress-induced hormonal responses in male and female rats (Minhas et al. 2016) could be in part mediated by greater reservoirs or production of these hormones in the pituitary and adrenal glands prior to puberty. Future studies will be needed to examine this question more directly. For instance, we have previously shown that prepubertal male rats show heighten adrenal corticosterone responses compared to adults 1 h after exposure to similar levels of ACTH (Romeo et al. 2014). Thus, as done previously, *in vivo* or *in vitro* approaches could be used to generate ACTH and corticosterone dose-response curves in prepubertal and adult rats using exogenous CRH and ACTH, for example, to further address these questions (Turkelson et al. 1981; Rivier and Vale 1983; Romeo et al. 2014).

## 5. Conclusion

Taken together, our data indicate significant age-dependent decreases in the levels of ACTH, and its precursor, POMC, in the pituitary and decreases in the levels of corticosterone in the adrenal glands of both male and female rats. Given the greater stress-induced HPA response in prepubertal compared to adult animals (reviewed in; (Green and McCormick 2016; Romeo 2018), these data suggest that greater hormonal concentrations in the pituitary and adrenal glands contribute to these developmental changes in hormonal stress reactivity. Thus, these data add to our growing understanding of the factors that mediate the pubertal changes in HPA function and call attention to the role that the peripheral glands of the HPA axis may play in these changes.

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## CRediT authorship contribution statement

**Rebecca B. Kann:** Conducted the research, Contributed to the writing and editing of the manuscript. **Russell D. Romeo:** Designed the experiments, Conducted the research, Contributed to the writing and editing of the manuscript.

## Declaration of competing interest

The authors declare that there is no conflict of interest, financial or otherwise.

## Data availability

Data will be made available on request.

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