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Acute stress imposed during adolescence has minimal effects on hypothalamic-pituitary-adrenal (HPA) axis sensitivity in adulthood in female Sprague Dawley rats

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Abstract

Adolescence is a developmental epoch marked by maturation of stress-responsive systems including the Hypothalamic-Pituitary-Adrenal (HPA) axis. Emerging evidence has found sex-specificity in the long term behavioral and neural effects of stressors experienced during this sensitive period, though most studies have utilized chronic stress exposures that span much of the adolescent period. Using Sprague-Dawley rats, we examined how a single exposure to inescapable footshock (80 shocks, 5s, 1.0 mA, 90 sec variable ITI) applied during early adolescence (PND 29-31) affected the corticosterone (CORT) response to a later restraint stress challenge in adulthood. We found that females, but not males, displayed a marginally enhanced CORT response when challenged with restraint in adulthood. To further probe intrinsic sensitivity of the HPA axis in adolescent stressed females, subsequent studies utilized exogenous CRH and ACTH challenges to probe sensitivity of the pituitary and adrenal glands respectively, demonstrating that neither gland appears to be sensitized to hormone challenge as a result of adolescent stress history in females. A final experiment examined negative feedback regulation of the HPA axis through systemic administration of dexamethasone, showing that corticosteroid receptor-mediated negative feedback mechanisms were also intact in females with a history of adolescent stress. Together, these findings report that intrinsic regulatory elements of the HPA axis are fully intact in females exposed to footshock in adolescence, and that adolescent exposure to footshock had appreciably modest long-lasting effects on HPA axis sensitivity. These findings are discussed within the general context of stress resilience and vulnerability.

Keywords

adolescence; stress; corticosterone; corticotrophin-releasing hormone; ACTH; sex differences; female

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Introduction

Adolescence is a transitional stage of development characterized by major neurobiological changes and heightened stress responsiveness (Dahl & Gunnar, 2009; Klein & Romeo, 2013). During this developmental epoch, stress exposure is associated with increased prevalence of affective disorders such as depression later in life (Conley & Rudolph, 2009), and studies have found sex-specific outcomes (Bourke & Neigh, 2011). One major component of the stress response that undergoes changes in adolescence is the hypothalamic-pituitary-adrenal (HPA) axis. The axis is initiated when corticotropin-releasing hormone (CRH) is secreted by cells residing in the paraventricular nucleus of the hypothalamus (PVN) inducing the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which in turn induces the adrenal glands to secrete glucocorticoids, specifically cortisol in humans and corticosterone in rats (CORT; see Spencer & Deak, 2017 for a recent review). CORT plays an important role in the stress response, exerting physiological effects throughout the body including mobilization of stored energy for immediate use, alteration of feeding and reproductive systems, and enhancing available glucose levels to brain regions in cognitive function (for review, see Sapolsky, Romero, & Munck, 2000). Importantly, glucocorticoids also contribute negative feedback, aiding shutoff of the HPA axis (Herman et al., 2003). In adolescent males, the HPA axis is functionally immature, and this has been observed in both the CORT and ACTH responses. For instance, the ACTH response to restraint was shown to be higher in adolescent rats at P30, P40, and P50 than in adults, and adult-like levels were achieved by 60 days of age, whereas peak CORT responses were adult-like by P40 (Foilb, Lui, & Romeo, 2011). However, the CORT response in adolescence was slower to resolve which may be indicative of immature negative feedback regulation of the axis (Foilb et al., 2011; Romeo, 2013; Romeo, Lee, & McEwen, 2004). This is consistent with other studies showing that male Sprague-Dawley adolescent rats acutely exposed to 90 minutes of restraint did not exhibit the post-stressor CORT decline at the 120 minute time point (30 minutes after the cessation of stress) that was seen in adults (Doremus-Fitzwater, Varlinskaya, & Spear, 2009), and the same delayed return to baseline was found after 30 min of restraint in females at P28 (Russell D Romeo et al., 2004).

Recently there has been much interest in how perturbations in adolescence can alter the development of stress-related systems and shape future stress reactivity, often termed developmental programming effects (Romeo, Patel, Pham, & So, 2016). The majority of studies have been performed using chronic stress over a period of weeks starting at various points throughout adolescence. For instance, an early study exposed male rats to either chronic variable stress (CVS) or chronic social stress (CSS) for four weeks starting at P28. They found that when the CVS group was tested on the elevated plus maze at P77, the CORT response remained high at 60 min whereas in the control and CSS groups it was returning to baseline (Isgor, Kabbaj, Akil, & Watson, 2004). In another study where adolescent Wistar rats were exposed to alternating restraint and social defeat from PND 37-49 then exposed to 5 minutes of forced swim on PND 53, the female, but not male, CORT response took longer to resolve (Bourke et al., 2013). Interestingly, the influence of adolescent stress challenges on adult HPA axis reactivity may not be exclusive to

psychological stressors, since chronic alcohol during adolescence yielded sensitized CORT responses in adulthood, an effect that was also sexually dimorphic (Vore, Doremus-Fitzwater, Gano, & Deak, 2017). Importantly, these outcomes bear some similarity to studies where adolescent-like responses have been shown to persist into adulthood, sometimes termed “locking in-like” effects (Fleming, Acheson, Moore, Wilson, & Swartzwelder, 2012; Spear & Swartzwelder, 2014). Nevertheless, since chronic administration of glucocorticoids can have deleterious consequences (Sternier & Kalynchuk, 2010), prolonged secretion of CORT in adolescence may put adolescents at greater risk for future disorders (McCormick & Green, 2013). Thus, HPA axis dysfunction that persists across the lifespan may be particularly detrimental.

In contrast to these studies, other work has demonstrated HPA-axis *hypo*activity specifically in female rats from the Wistar line with a history of chronic adolescent stress. Chronic mixed-modality stressors, which includes both social and non-social stressors, from P37-49 led to blunted secretion of CORT in response to restraint stress in adulthood in females but not in males (Bourke & Neigh, 2011). Another study using CVS from P45-58 that then exposed Sprague Dawley rats to 30 minutes of forced swim during adulthood corroborated this finding, and also found a reduced ACTH response (Wulsin, Wick-Carlson, Packard, Morano, & Herman, 2016). Consistent with this, the observation of reduced AVP expression led to the conclusion that adolescent stress may reduce excitatory drive within the HPA axis. Although these studies did not conduct detailed time course analyses, the timing of the measures would suggest that peak responding was lower in subjects that experienced chronic stress in adolescence. Thus, it is clear that chronic stress experienced in adolescence can have lasting effects on HPA-axis reactivity particularly in females, with some studies reporting enhanced HPA axis responding and others demonstrating the opposite. Careful consideration of subject parameters, including precise consideration of age during which the stress challenge is imposed and prior stress history, will be necessary in interpreting these differing outcomes. Thus, development of acute stress models that can be imposed during more discrete subperiods of adolescence (i.e., pre-pubertally, postpubertally, etc) would be advantageous.

Though most studies examining the impact of stress imposed during adolescence have utilized chronic stress models, we recently performed a series of studies examining the impact of exposure to a single, intense stress challenge imposed during early adolescence (P29-30) on behavioral and neuroendocrine responses to stress later in adulthood (Lovelock & Deak, 2019). A single intense stress challenge was chosen because it produces a robust stress response that mimics changes more typically associated with chronic stress, but has the added advantage of requiring only a single exposure. As a result, the age of exposure can be more readily manipulated with a single session of footshock, in contrast to CVS models that require exposure periods of 10-21 days, spanning the majority of the rodent adolescent period. This is important because HPA-axis abnormalities have been consistently found in the pre-pubertal period (early adolescence), and this developmental epoch may represent a sensitive period of vulnerability (Romeo, 2010). Our prior studies showed that exposure to a single session of intermittent footshock (80 shocks, 5 sec each, 90 sec variable inter-trial interval) produced sex-specific outcomes, with males exhibiting enhanced anxiety in the light-dark box whereas females did not. Females, however, showed a strong tendency toward

increased basal CORT if they had experienced adolescent footshock, suggesting that female adolescents may be more vulnerable to HPA axis perturbations than males (Lovelock & Deak, 2019). However, these studies, and most studies in the literature, looked at the CORT response at a single timepoint, making it difficult to determine whether these changes reflect alterations in peak responding or might relate to HPA-axis negative feedback. Furthermore, our recent work showed that repeated ethanol exposure during adolescence led to enhanced CORT reactivity in adulthood exclusively in females (Vore et al., 2017).

Thus, the goal of these experiments was to systematically probe HPA-axis sensitivity after exposure to a single session of footshock in adolescence. The goal of Experiment 1 was to test whether adolescent footshock exposure would alter the HPA axis response to a mild stress challenge during adulthood (60 min of restraint) in both sexes. This approach had the advantage that restraint lends itself to detailed time course analyses, thereby ensuring that potential sex differences in the kinetics of the CORT response could be more aptly addressed. We hypothesized that females exposed to adolescent footshock would display a sensitized CORT response, and that this effect may be driven by adaptation within the HPA axis such as a sensitized pituitary response to CRH or a sensitized adrenal response to ACTH. Thus, Experiments 2 and 3 utilized peripheral injections of corticotrophin-releasing hormone (CRH) or adrenocorticotrophic hormone (ACTH) to determine whether altered HPA axis reactivity in females was occurring at the levels of the pituitary and adrenal glands, respectively. Finally, in order to determine if the HPA-axis negative feedback system was impaired as a result of adolescent footshock, we utilized dexamethasone, a synthetic glucocorticoid that engages negative feedback mechanisms in the HPA-axis, to determine if CORT release would still occur in response to restraint. In addition to CORT and ACTH, we also measured plasma progesterone concentrations as a secondary index of adrenal stress reactivity, since progesterone lies within the biosynthetic pathway that ultimately leads to CORT secretion and is similarly induced by injection of ACTH and CRH (Hueston & Deak, 2014; Torres, Ruiz, & Ortega, 2001).

Methods

Subjects

All experiments used Sprague-Dawley rats bred and born in our colony from breeders originally acquired from Envigo/Harlan. Litters were culled to a maximum of 10 pups and no litter had less than 3 pups of each sex. Rats were weaned and pair-housed with non-littermates at PND 21-22, given access to food and water *ad libitum*, and were provided wooden chew sticks for enrichment. Colony conditions were maintained at 22±1°C with 12:12 light–dark cycle (lights on 06:30 h). Rats were handled for 3-5 min on each of two days before experimentation as adolescents, and again one day prior to adult testing. In all experiments, experimental groups contained no more than 1-2 pups from the same litter to avoid litter effects, and in the majority of cases each litter was only represented once. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Binghamton University and animals were treated in accordance with PHS policy.

Footshock

In all experiments, rats were exposed to one session of footshock during the light cycle (typically between 0900-1200) between the ages of P29-31 as noted below. This age range was chosen as an early adolescent window that predictably preceded pubertal onset (typically ~P35 for females and ~P42 for males). At the appointed age, rats were exposed to 80 inescapable footshocks (1.0 mA, 5 s each, 90 s variable inter-trial interval). The footshock chamber measured 30.5 (L) × 26.5 (W) × 33 (H) cm (Habitest Chamber, Model H10-11R-TC-SF, Coulbourn Instruments, Allentown, PA, USA). The side walls of the chamber were constructed of stainless steel except the front doors which were constructed of clear Plexiglas. The floor consisted of steel rods through which a scrambled shock from a shock generator (LABLINC Model H01-01, and Precision Animal Shocker Model H13-15, Coulbourn Instruments, Allentown, PA, USA) could be delivered. The chambers were sound-attenuating and illuminated by a 20-W white light bulb and background noise was provided by individual ventilation fans.

Restraint and tail blood collection

Rats were restrained in Plexiglas tubes (length = 20.4 cm, inner diameter (ID) = 5.0 cm) with ample holes for ventilation for either 60 or 90 minutes. The restraint stressor was devoid of any active immobilization, limb/tail tethering, or compression, and allowed sufficient movement so that animals could rotate (barrel roll) within the tube but did not allow for them to turn around head to tail. At collection time points, blood (50-100 µl) was collected with gentle massaging of the tail into 0.5 ml tubes and samples were immediately placed on ice. All blood samples were collected within 2 min to ensure serum measures of CORT reflected ambient levels untainted by the stress of the blood sampling procedure itself. Rats were returned to their home cages immediately afterwards or remained in restraint as dictated by group assignment. Serum was separated for 15 min at 3220 g in a refrigerated centrifuge and frozen at -20°C until time of assay.

Trunk blood collection

Trunk blood was collected in EDTA-coated vacutainers containing 50 µl aprotinin (Cat No: 190779; MP Biomedicals, Solon, OH). Plasma was separated in a refrigerated centrifuge and frozen at -20°C until time of assay.

Measurement of Hormones

Total serum CORT and progesterone concentrations were measured using commercially available Enzyme Immuno Assay (EIA) kits (Enzo Life Sciences; Farmingdale, NY) according to manufacturer's instructions, with one exception. Samples were heat-inactivated to denature endogenous corticosteroid binding globulin (CBG) via immersion in a 75°C water bath for 60 min (Buck et al., 2011; Hueston and Deak, 2014). Prior assays show this procedure produces superior denaturation of CBG than the steroid displacement reagent provided in the CORT ELISA kit (see Spencer & Deak, 2017 for discussion), and assay validation studies demonstrated no adverse effect of heat denaturation on plasma PROG (T. Deak, unpublished observations). Inter-assay coefficients of variation were 3.67% for CORT and 8.45% for progesterone. Quantitative determination of plasma ACTH was assessed by a

commercially available ACTH enzyme-linked immunosorbent assay (ELISA) kit (Cat No: M046006; MD BioSciences, St. Paul, MN) according to the manufacturer's instructions. Undiluted serum samples were assayed after a single freeze-thaw cycle.

Experiment 1 procedure

Recent studies from our lab have shown that adolescent exposure to chronic ethanol exposure led to sensitized CORT responses in adulthood, an effect that was specific to females (Vore et al., 2017). In addition, adolescent (females but not males) exposed to footshock displayed moderately elevated basal CORT concentrations in adulthood, providing evidence that footshock in particular may produce long-lasting HPA dysregulation in females (Lovelock & Deak, 2019). Thus, the goal of Experiment 1 was to test whether adolescent footshock would alter restraint-induced CORT and progesterone responses in both sexes. Restraint was selected as a stress challenge during adulthood because the peak CORT response is typically lower than that evoked by other stress challenges such as forced swim (Hueston et al., 2011), allowing for bi-directional sensitivity when testing sensitization effects. In addition, restraint allows for easy acquisition of multiple blood samples, affording greater temporal clarity in the kinetics of the corticosterone response using a within subjects design. Adolescent male and female rats (N = 16 per sex) were either exposed to footshock in early adolescence (P29-30) or remained in their home cages as controls. For feasibility purposes, male and female rats were run in separate cohorts and thus could not be compared statistically. After the adolescent stress challenge, all rats remained undisturbed in the colony until adulthood. As adults (P80-P82), all rats were exposed to 60 minutes of restraint as described above and then returned to their homecage. Tail blood samples were collected immediately after placement in the restraint tube to assess baseline CORT (described graphically as the 0 min sample), with subsequent samples collected 15, 30, 60, and 120 minutes after stress onset and analyzed for CORT and progesterone concentrations (Figure 1A). Thus, the 120 min time point reflected a (60 min) post-stress recovery sample to assess resolution of hormonal responses.

Experiment 2 procedure

The goal of Experiment 2 was to determine whether sensitivity of the pituitary gland would be altered in female rats exposed to footshock during adolescence. To do this, adolescent female rats (N = 32, n = 8/group) were either exposed to footshock in early adolescence (P29-31) or remained in their home cages and subsequently remained undisturbed in the colony until adulthood. Adult (P79-P81) rats were then injected with either 0 or 1.0 µg/kg CRH (i.p.; Catalog # C3042, Sigma-Aldrich, St. Louis, MO) dissolved in sterile physiological saline (0.9%, TEKnova, Hollister, CA). This dose was selected based upon recent findings from our lab demonstrating an intermediate-level ACTH and CORT response when injected peripherally (Hueston & Deak, 2014). Studies to date have not investigated the differences in magnitude of the response to CRH injection between males and female rats, however in humans a 1.0 µg/kg did not elicit differential responding in males and females (Gallucci et al., 1993). One hour after injection, rats were then rapidly decapitated under stress-free conditions and trunk blood was collected for measurement of ACTH and CORT (Figure 2A).

Experiment 3 procedure

This experiment was designed to test sensitivity of the adrenal gland to exogenous ACTH challenge in female rats exposed to footshock during adolescence using a 2×2 design. To do this, adolescent female rats (N = 32, n = 8/group) were either exposed to footshock in early adolescence (P29-30) or remained in homecages and subsequently remained undisturbed in the colony until adulthood. Adult (P74-P76) rats were then injected with 0 or 2.5 IU/kg ACTH (i.p.; Catalog # A6303, Sigma-Aldrich, St. Louis, MO) dissolved in sterile physiological saline (0.9%, TEKnova, Hollister, CA) as the vehicle. Thirty minutes post-injection, rats were then rapidly decapitated under stress-free conditions and trunk blood was collected for measurement of ACTH, CORT and progesterone (Figure 3A). This dose and timing was selected based upon recent findings from our lab indicating a robust yet submaximal CORT response in male Sprague Dawley rats after this dose (Hueston & Deak, 2014).

Experiment 4 procedure

Since there were no effects of hormone-mediated stimulation of either the pituitary gland (Experiment 2) or the adrenal glands (Experiment 3), we performed one final test of HPA axis regulation to assess negative feedback regulation. Thus, Experiment 4 utilized a dexamethasone suppression test since injection of dexamethasone engages negative feedback mechanisms and suppresses the production and release of CORT (Hueston & Deak, 2014; Riegle & Hess, 1972). Adolescent female rats (N = 32, n = 8/group) were either exposed to footshock in early adolescence (P29-31) or remained in homecages and subsequently remained undisturbed in the colony until adulthood. Adult (P77-P80) rats were then injected with 50 µg/kg dexamethasone (subcutaneous; catalog # D1756, Sigma-Aldrich, St. Louis, MO) dissolved in 50% propylene glycol (catalog # P4347, Sigma-Aldrich, St. Louis, MO) and 50% sterile physiological saline (0.9%, TEKnova, Hollister, CA) or vehicle, and 90 min later were placed in restraint for 30 min. Rats were then rapidly decapitated under low stress conditions and trunk blood was collected for measurement of ACTH, CORT and progesterone (Figure 4A). This dose and timing was selected in order to maximally suppress the CORT response and was based upon previous findings from our lab (Hueston et al., 2014).

Statistics

All analyses were conducted in Statistica with ANOVAs appropriate for the particular design, as noted in the individual studies below. Post hoc analyses were performed using the Fisher's LSD method when a significant interaction was found. Criterion for rejection of the null hypothesis was always $p < 0.05$.

Results

Experiment 1.

Repeated measures ANOVAs using adolescent stress history as a between subjects variable and time of sample collection as a variable were run separately for each sex. In females, a repeated measures ANOVA found a main effect of time point on restraint-induced CORT

[F(4, 56)=86.28, $p<0.0001$], with post-hoc analysis indicating that the 15, 30, and 60 minute time points showed an increase from baseline ($p<0.0001$ in all cases) and a reduction at the 120 minute recovery time point ($p<0.01$; figure 1B). While not significant, there was a strong trend for an effect of adolescent footshock [F(1, 14)=3.42, $p=0.086$]. To further probe this effect, Area Under the Curve analysis was performed, which also yielded a marginally significant effect of adolescent footshock ($p<0.01$; data not shown). No effects were found in progesterone concentrations of females [F(4, 56)=2.08, $p>0.05$]. In males, there was a main effect of time point on restraint-induced CORT [F(4, 56)=78.32, $p<0.0001$] with increased levels at the 15, 30, and 60 minute time points ($p<0.0001$ in all cases) and a return to baseline levels at 120 minutes (figure 1C). Also in males, there was a main effect of time point on progesterone [F(4, 56)=32.92, $p<0.0001$] that did not fully resolve by 120 minutes ($p<0.0001$; figure 1E). To better detect changes in the peak response, we also conducted 2×3 ANOVAs focused on the 15, 30, and 60 min timepoints (i.e., excluding baseline and recovery time points). Again, there was a strong trend for a main effect of adolescent footshock in females [F(1, 14)=3.58, $p=0.079$], but no difference between timepoints [F(2, 28)=1.47, $p>0.05$]. No effects were found on progesterone levels. In males, there was a main effect of timepoint [F(2, 28)=21.82, $p<0.001$] with the 15 minute timepoint being lower than the 30 and 60 min timepoints (p 's < 0.001), while there was no effect of adolescent footshock. Progesterone concentrations mirrored those of CORT [F(2, 28)=11.08, $p<0.01$], again increasing after the 15 minute timepoint (p 's < 0.01) but adolescent shock had no effect.

Experiment 2.

A 2×2 ANOVA found a main effect of CRH injection on ACTH [F(1, 28)=5.13, $p<0.05$] and a significant interaction between adolescent stress condition and adult CRH challenge [F(1, 28)=4.37, $p<0.05$] (Figure 2B). Post-hoc analysis revealed that ACTH concentrations were significantly lower in the adolescent-shocked vehicle injected group as compared to all other groups ($p<0.05$ in all cases). As expected, CRH injection significantly increased CORT [F(1, 28)=11.34, $p<0.01$] but there was no effect of adolescent shock (figure 2C). Progesterone was not affected by adolescent footshock nor CRH injection.

Experiment 3.

As expected, use of a 2×2 ANOVA found a main effect of exogenous ACTH challenge on plasma concentrations of ACTH [F(1, 28)=25.61, $p<0.0001$] and CORT [F(1, 28)=151.29, $p<0.0001$]. There was also a main effect of ACTH injection on progesterone [F(1, 28)=4.84, $p<0.05$] as well as a significant interaction between adolescent stress history and adult ACTH challenge [F(1, 28)=8.19, $p<0.01$]. Post-hoc analysis revealed that the adolescent homecage-adult homecage group had less progesterone as compared to all 3 other groups (p 's < 0.05).

Experiment 4.

Analysis with a 2×2 ANOVA found main effects of dexamethasone injection on ACTH [F(1, 28)=13.18, $p<0.01$] and CORT [F(1, 28)=37.57, $p<0.0001$], with dexamethasone substantially reducing plasma concentrations of both ACTH and CORT relative to vehicle-

injected, restrained subjects No significant interactions between adolescent stress history and adult dexamethasone injection were observed. There were no effects on progesterone.

Discussion

These experiments tested whether acute stress challenge incurred during early adolescence would produce alterations in intrinsic HPA axis regulation that persist into adulthood. Adolescent footshock revealed subtle, yet important effects on the CORT response to restraint (Experiment 1) in adulthood that were sex specific, similar to our prior findings after ethanol administration (Vore et al., 2017). Although the overall effect observed here was statistically marginal, it should be noted that the difference in peak CORT was approximately 20 µg/dl, which is more than sufficient to increase corticosteroid receptor occupancy (Deak et al., 1999; Fleshner et al., 1995). The adolescent footshock manifested in adulthood as higher CORT at 60 minutes of restraint when control subjects were beginning to decline from peak, similar to the extended timeline of the CORT response commonly seen in adolescence (Foilb et al., 2011). Thus, this suggested that an acute stress challenge imposed during early adolescence might enhance CORT responses in adulthood, only in females.

In comparing our findings with the broader literature, the parameters of each particular experiment need to be considered carefully. While there are several examples where stress in adolescence resulted in an enhancement of the adult CORT response (Lepsch et al., 2005; Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007), this contrasts with studies where chronic stress was administered during adolescence and found CORT to be *hyporesponsive* later in life (Bourke & Neigh, 2011; Wulsin et al., 2016). Importantly, footshock in our studies was applied specifically in early adolescence at a time point that is likely prepubertal for both sexes (P29-P31), whereas the aforementioned studies took place across multiple days in mid-late adolescence (Spear, 2015). Thus, it is possible that outcomes are dependent upon whether stressors are imposed during sensitive periods specific to early versus late adolescence. Additionally, other aspects of the stress history in subjects from other published studies may play an important role (Spencer & Deak, 2017). In the above examples, in cases of reduced CORT responding the subjects were obtained via ordering timed pregnant dams. In cases of enhanced CORT responding, subjects were bred in-house and thus did not have a pre/perinatal stress experience. Shipping stress during the prenatal period has been shown to have deleterious effects and to modify responses to drug treatments (Ogawa, Kuwagata, Hori, & Shioda, 2007; Wiley & Evans, 2009), and may also be responsible for modulating the programming effects instantiated by stress exposure in adolescence. Thus, it is important to determine if the additional experience of prenatal or early life shipping stress could be affecting study outcomes and interpretations, making those studies a test of stressors at multiple developmental time points rather than just during adolescence, as we have done in the present studies.

In Experiments 2 and 3 we conducted follow up studies to determine whether intrinsic sensitivity of the HPA axis would be altered by exposure of females to adolescent footshock. Our approach was to probe HPA axis sensitivity at the levels of the pituitary and adrenal glands using CRH and ACTH challenges, respectively. Adolescent stress did not affect HPA-

axis responses to CRH or ACTH challenges, suggesting that the pituitary and adrenal glands remained equally sensitive to hormone challenge in females with a history of adolescent stress. Yet, a small but statistically reliable reduction in ACTH was observed in shocked rats challenged with CRH, which may be indicative of altered CRH receptor expression and or function. This intriguing finding should be pursued in future studies. Because these exogenous hormone challenges occurred in the absence of an accompanying stress challenge, their findings might suggest alterations in brain regions that project to the PVN such as catecholaminergic input from brainstem autonomic nuclei or glutamatergic projections from the bed nucleus of the stria terminalis (BNST) where input from multiple brain regions including the amygdala and ventral subiculum are consolidated (Herman, Cullinan, & Herman, 1997). The BNST has been implicated in modulating HPA axis activation after chronic stress as lesions in the anteroventral or posterior medial BNST potentiated the HPA axis response after CVS (Choi et al., 2007; Choi, Evanson, et al., 2008; Choi, Furay, et al., 2008). We previously found that adolescent stress induced a small but significant increase in medial amygdala c-Fos activation in response to forced swim which may also be relevant for HPA axis alterations (Lovelock & Deak, 2019). MeA projections to the BNST primarily target GABA-ergic neurons (Crestani et al., 2013; Cullinan, Herman, & Watson, 1993), and future studies will be needed to determine if sensitization might be reflected by altered activity within this circuit.

The final experiment examined the integrity of HPA axis negative feedback via a DEX suppression test. The point of this test is to activate corticosteroid receptors with exogenous glucocorticoid, thereby engaging negative feedback mechanisms and thus suppressing the production of endogenous CORT. DEX exerts its negative feedback effects through activation of GR in CNS structures including the hippocampus, PVN and other sites projecting to the PVN, as well as through direct action in the pituitary and adrenal glands (Cole, Kim, Kalman, & Spencer, 2000; Kalin, Weiler, & Shelton, 1982; Miller et al., 1992). In subjects with impaired negative feedback mechanisms, one would predict that DEX would be less effective at inhibiting CORT release, which would then manifest as higher CORT concentrations in the blood relative to subjects with no history of adolescent stress. Importantly, DEX suppression has been shown to be impaired after adult exposure to inescapable tailshock, though these effects were not evaluated within a developmental framework (O'Connor et al., 2003). In Experiment 4, we found that DEX successfully, and nearly completely, reduced CORT levels in both groups, indicating that CORT negative feedback remained intact after adolescent stress exposure. Thus, feedback regulation mediated through GR appears to be fully intact in females with a history of adolescent footshock exposure. With that said, the present experiment utilized a dose of DEX that showed near-complete shutoff of the HPA axis. We cannot rule out the possibility that a broader dose-response function might reveal more subtle differences in HPA axis negative feedback regulation after adolescent footshock. Furthermore, since peripheral DEX administration likely exerts its effects on feedback regulation largely through actions in the pituitary and adrenal glands (Cole, Kim, Kalman & Spencer, 2000), it remains possible that central mechanisms of HPA axis feedback regulation may be disrupted by adolescent footshock exposure. These issues will require future studies. Moreover, the efficacy of DEX to shut off the axis does not preclude the possibility that more subtle alterations in

glucocorticoid receptor expression or function might result from adolescent stress exposure in females. Indeed, our prior studies found no difference in GR or MR expression levels after adolescent footshock when receptor expression was assessed via real time RT-PCR (Lovelock & Deak, 2019). Unfortunately, prior analyses did not take into account multiple isoforms of GR, including the most common subtype GR α and less commonly expressed inhibitory GR β , which are produced through alternative splicing (i.e., the primer sequences targeted a conserved region of the coding sequence common to both receptor subtypes). It has been demonstrated previously that neonatal handling can alter expression of these subtypes through epigenetic programming (Weaver et al., 2004). Thus, it is possible that changes in receptor subtype expression could be induced by adolescent stress, and this could be examined through using subtype-specific primers.

One significant difference between males and females is that they differ in both ambient, baseline CORT concentrations under non-stressed conditions, and females display a peak CORT response that is nearly double that of males. These differences occur largely due to the modulatory effects of estrogens on HPA axis output (see Oyola & Handa, 2017 for a recent review). The studies here provide evidence for both of these intrinsic sex differences (eg., Figure 1). In males, baseline CORT typically ranges between 0-5 $\mu\text{g/dl}$, depending on the mode of sample collection and the type of assay utilized to measure CORT. In contrast, females in the present experiments displayed baseline CORT ranging as high as 12 $\mu\text{g/dl}$ in experiment 1 to 30 $\mu\text{g/dl}$ in 30 min after saline injection in Experiment 3. Baseline CORT levels in females have been shown to have much greater variance across the diurnal rhythm as compared to males, varying from approximately 7-50 $\mu\text{g/dl}$ between the trough and peak, with a sharp rise occurring 4 h after lights on (Chun, Woodruff, Morton, Hinds, & Spencer, 2015). Further, peak values are dependent on estrous cycle and were shown to range from 29-47 $\mu\text{g/dl}$ between estrus and proestrus, respectively (Atkinson & Waddell, 1997). Additionally, in the case of Experiments 2 and 3, control subjects were injected with saline either 30-60 minutes before blood sampling, and saline injections have been shown to increase CORT levels within this time frame (Willey, Anderson, Morales, Ramirez, & Spear, 2012). Although CORT values cannot be compared directly between males and females in Experiment 1, it is notable that peak plasma CORT in females was more than double that of males. One intriguing explanation for the female-specific enhancement of CORT release, therefore, might be that females display a much more profound CORT response to footshock during adolescence. By this logic, one would predict that augmenting the CORT response in males during adolescence to match the response in females might elicit similar enhancement of CORT later in life. We anticipate studies to address this issue in the near future.

It should be noted that serum/plasma PROG concentrations observed here fell within a typical normative range of ~1-15 ng/ml, which is consistent with our prior studies in both males and females (eg., Arakawa, Arakawa, Hueston, & Deak, 2014; Gano, Doremus-Fitzwater, & Deak, 2017; Hueston & Deak, 2014; Lovelock & Deak, 2019). However, other studies have reported notably higher plasma concentrations of PROG ranging up to ~30 ng/ml at peak (Green, Nottrodt, Simone, & McCormick, 2016; Romeo, Bellani, & McEwen, 2005; Romeo et al., 2004). Although the reasons for the lower PROG observed in the present studies are not clear, it should be noted that our work has routinely utilized commercially-available Enzyme-based ImmunoAssays (EIA kits) rather than more traditional

Radioimmunoassays (RIA) to assess plasma hormone concentrations. Other subtle differences in sample collection and processing might also contribute to these differences. Finally, endocrine reactivity is known to vary as a result of the distributor from which rats are derived, and as a result of shipping from commercial rodent facilities (Laroche, Gasbarro, Herman, & Blaustein, 2009; see Spencer & Deak, 2017 for discussion). While these differences do not offer a definitive explanation for the lower PROG response observed in the present studies, variations in peak response are not uncommon for endocrine studies.

Several limitations of the present studies need to be considered. First, estrous cycle was not taken into account. Indeed, one study found that chronic social isolation and instability stress from P30-45 resulted in an enhanced CORT response to forced swim in females only if they were in diestrus (Mathews, Wilton, Styles, & McCormick, 2008). Because females were exposed to footshock prior to puberty, estrous cyclicity is a concern predominantly at the time of restraint exposure in adulthood. Due to concerns about how daily vaginal lavage might impact stress reactivity, we elected not to track estrous cycle in the present studies. Nevertheless, it is possible that variation due to estrous stage contributed greater variability to the magnitude of the effect, thereby yielding a marginally significant enhancement of CORT. To address this, future studies should utilize a design in which estrous cyclicity is tracked. Second, in the CRH and ACTH challenge experiments, single doses and timepoints were chosen for feasibility purposes based on our previously published dose-response functions (Hueston & Deak, 2014). However, the injections in the present experiments were based on previous studies that used effective doses in males due to a highly limited literature in females. As a result, we observed substantially elevated CORT concentrations in females after the ACTH injection (Figure 3) which may have produced a ceiling effect that complicates interpretation of results. Thus, future studies may be necessary to systematically test a range of doses and time points in order to more comprehensively assess sensitivity of the pituitary and adrenal glands in females with a history of adolescent footshock. Despite this, one contribution of the present studies is that they begin to establish a normative range of dose-effect relationships for classic pharmacological manipulations of the HPA axis in females. Lastly, in Experiment 2, baseline ACTH levels in controls that received a saline injection were found to be lower in subjects that experienced adolescent footshock. Few studies report on baseline levels of ACTH, and even when examining downstream targets of ACTH the outcomes vary, with reports ranging from subtle decreases in CORT after CVS (Taylor, Taylor, & Koenig, 2013) to increased basal CORT specifically in females after repeated restraint stress in adolescence (Barha, Brummelte, Lieblich, & Galea, 2011). Regardless, this effect did not replicate in Experiment 3 despite equivalent saline injection volumes, suggesting the effect may have been spurious. Despite these limitations, the present series of studies provide important guidance on future directions towards understanding the female-specific HPA axis reactivity after adolescent stress.

From a global perspective, glucocorticoid hormones have effects on nearly every cell in the body, with roles ranging from stress responding to cardiovascular function to moderating maturation and neural plasticity (McCormick, Mathews, Thomas, & Waters, 2010; Wada, 2008). The present studies found evidence of a modestly increased CORT response yet the HPA axis response to direct hormone challenge remained intact. Future experiments might examine other mechanisms that could account for enhanced CORT responses such as

increased input from neural circuits that regulate the axis or altered descending neural regulation of adrenal CORT release (Engeland & Arnhold, 2005). Importantly, this finding was specific to females, and as sex differences in the CORT system have been implicated in sex-specific vulnerability to psychopathology, it highlights the importance of pursuing female-specific approaches not only in basic research but potentially in therapeutic settings as well. There are strong trends in the literature indicating the importance of early life adversity, and adolescence in particular, as periods for increased vulnerability to the development of psychological disorders (Dahl & Gunnar, 2009; Tottenham & Galván, 2016). Indeed, one can readily find multiple special issues of journals dedicated specifically to that purpose. Although the principle findings of the present studies are negative, these well-controlled, carefully-executed experiments provide an important counterweight to the extant literature by demonstrating that female rats exposed to intense, acute stress during adolescence retain largely normal HPA axis integrity into young adulthood. In this way, the present findings contribute an important piece of the puzzle toward a broader understanding of how stress-related experiences might moderate risk for future dysfunction.

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Highlights

- Prior studies have shown sexually dimorphic responses to adolescent stress
- The HPA axis reacted normally to CRH or ACTH challenge after adolescent stress
- A dexamethasone suppression test suggested intact negative feedback regulation
- Acute stress during adolescence may not manifest in HPA axis dysregulation
- These findings contrast with long-lasting behavioral effects observed in males

Figure 1.

Experiment 1 design (A). Male and female subjects were exposed to footshock in early adolescence. In adulthood, they were then exposed to 60 minutes of restraint, and tail blood was collected at key time points for CORT (B and C) and progesterone (D and E) plasma concentrations. A trend for a main effect of adolescent shock was found in the CORT response in females.

Figure 2.

Experiment 2 design (B). Female subjects were exposed to footshock in early adolescence, and in adulthood were exposed to a CRH challenge. Serum was collected and analyzed for concentrations of key HPA axis hormones. The vehicle control group had reduced ACTH at baseline (B), CRH injection induced an increase in CORT regardless of stress history in adolescence (C), and no effects were seen in progesterone (D). * indicates a main effect of CRH injection, and different letters indicate differences between groups.

Figure 3.

Experiment 3 design (B). Female subjects were exposed to footshock in early adolescence, and in adulthood were exposed to an ACTH challenge. Serum was collected and analyzed for concentrations of ACTH (B), CORT (C), and progesterone (D). ACTH injection induced increases in ACTH and CORT concentrations regardless of adolescent stress history, while progesterone was lower in the control group that did not receive shock or ACTH compared to all other groups. * indicates a main effect of ACTH injection, and different letters indicate differences between groups.

Figure 4.

Experiment 4 design (B). Female subjects were exposed to footshock in early adolescence, and in adulthood were exposed to a dexamethasone suppression test. Serum was collected and analyzed for concentrations of ACTH (B), CORT (C), and progesterone (D). DEX successfully suppressed both ACTH and CORT in the adolescent stress history group, indicating that HPA axis negative feedback remained intact. * indicates a main effect of DEX injection.