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Chronic Restraint Stress in Adolescence Differentially Influences Hypothalamic-Pituitary-Adrenal Axis Function and Adult Hippocampal Neurogenesis in Male and Female Rats

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ABSTRACT: Previous studies have shown a relationship between adversity in adolescence and health outcomes in adulthood in a sex-specific manner. Adolescence is characterized by major changes in stress-responsive regions of the brain, including the hippocampus, the site of ongoing neurogenesis throughout the lifespan. Prepubertal male and female rats exhibit different acute reactions to chronic stress compared to adults, but less is known about whether these stress-induced changes persist into adulthood. Therefore, in this study, we investigated the effects of chronic, intermittent stress during adolescence on basal corticosterone levels, dentate gyrus (DG) volume, and neurogenesis in the hippocampus of adult male and female Sprague-Dawley rats. Adolescent male and female rats were either restrained for 1 h every other day for 3 weeks from postnatal days (PDs) 30–52 at unpredictable times or left undisturbed. All rats received a single injection of bromodeoxyuridine (BrdU; 200 mg/kg) in adulthood on PD70 and were perfused 3 weeks later. Brains were processed for Ki67 (endogenous marker of cell proliferation) and BrdU (to estimate effects on cell survival). In addition, blood samples were taken during the restraint stress period and in adulthood. Results show that males and females exhibit different corticosterone responses to chronic stress during adolescence and that only adult female rats exposed to stress during adolescence show higher basal corticosterone levels compared to nonstressed controls. Furthermore, stressed females showed a reduced number of proliferating and surviving cells in the DG in adulthood compared to nonstressed same-sex controls. The majority of BrdU-labeled cells were co-labeled with NeuN, an endogenous marker of mature neurons, indicating that neurogenesis was decreased in the DG of adult female rats that had undergone chronic restraint stress in adolescence. Although male rats were more responsive to the chronic stress as adolescents showing higher corticosterone levels and reduced body weight, as adults they showed a slight increase in cell survival and no effect of adolescent stress on basal corticosterone levels. These results suggest that stress during adolescence can have effects on hypothalamic-pituitary-adrenal axis function and hippocampus plasticity in adulthood, particularly in female rats. © **2010 Wiley Periodicals, Inc.**

KEY WORDS: cell proliferation; cell survival; sex difference; corticosterone; adolescence

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INTRODUCTION

Stressful experiences that occur during the adolescent period have a profound impact on the health and psychological functioning of the individual (Spear, 2000; Andersen, 2003; McEwen, 2003). For example, exposure to stress and adverse experiences during adolescence exacerbate the predisposition of children to neuropsychiatric disorders in adulthood in a sex-specific manner (Penza et al., 2003; Lenze et al., 2008). During adolescence the brain undergoes extensive maturation and shows a remarkable degree of plasticity both in structure and function (Romeo and McEwen, 2006). Furthermore, this developmental time period is characterized by major changes in the stress-responsive regions of the brain, including the hippocampus (Crews et al., 2007), a structure implicated in cognition, psychiatric disorders, and the site of ongoing neurogenesis throughout the lifespan. Therefore, it is believed that adolescence is a period of increased vulnerability and that stress-induced perturbations in the developing adolescent brain may contribute to altered functioning later in life.

The adolescent period is further characterized by the maturation of the hypothalamic-pituitary-adrenal (HPA) axis. Exposure to stressful experiences during adolescence can have profound effects on the development of the HPA axis, which can be long-lasting. Specifically, studies in rodents indicate that exposure to stress during adolescence can increase reactivity to subsequent stressors in adulthood, as assessed by higher corticosterone and adrenocorticotropic hormone levels in males in response to stress but not at baseline (Isgor et al., 2004) and higher corticosterone levels in females in response to stress (Pohl et al., 2007). Furthermore, adolescent male and female rats show prolonged corticosterone release in response to a stressor compared to adult rats (Romeo et al., 2004a,b; Viau et al., 2005; Romeo and McEwen, 2006) and adolescent male rats do not habituate to chronic stressors unlike adult rats who eventually show a blunted corticosterone response to chronic stressors (Gomez et al., 2004). Therefore, it seems likely that adolescents are at a greater risk for the deleterious effects of stress on HPA axis functioning than adults.

Exposure to stressful experiences during adolescence has profound long-lasting effects on a number of behavioral and neural outcomes. For instance, in male rodents, exposure to stress during adolescence alters stress reactivity (Isgor et al., 2004), cognitive ability (Isgor et al., 2004; Avital and Richter-Levin, 2005; Tsoory and Richter-Levin, 2006), emotionality (Pohl et al., 2007), and hippocampal structure (Isgor et al., 2004) in adulthood. Less work has been done in female rodents, and very few studies have directly compared males and females. However studies have shown that exposure to chronic mild stress during adolescence does alter anxiety and depressive-like behavior in female rats but not in male rats (Pohl et al., 2007). Furthermore, it has been suggested that adult females show a different sensitivity and coping mechanism for stress compared to adult males (Dalla et al., 2008), and there are sex differences in hippocampal dendritic atrophy and adult neurogenesis in response to stress in the adult (Galea et al., 1997; Falconer and Galea, 2003). Thus, it is conceivable that females may show a different vulnerability for stressful experiences during adolescence compared to males and may exhibit an altered spectrum of outcomes in response to adolescent stress in adulthood.

Exposure to chronic stress in adolescence and adulthood can alter the morphology of the hippocampus. For example, exposure to unpredictable physical and psychological stressors during adolescence resulted in decreased volume of CA1, CA3, and dentate gyrus (DG) regions of the hippocampus in male rats (Isgor et al., 2004). Interestingly, the effects of stress during adolescence may be permanent, unlike in adulthood where many effects of stress on the hippocampus are reversible with time (Girotti et al., 2006). In adulthood, exposure to stress increases cell death in the hippocampus, reduces hippocampal volume, and differentially affects neurogenesis in the hippocampus of male and female rodents. Progenitor cells in the DG of the hippocampus retain the ability to proliferate into neurons during adulthood in most mammalian species studied including humans (Eriksson et al., 1998; Gould et al., 1999). Specifically, adult males show suppressed cell proliferation and cell survival in response to acute and chronic stress, respectively, whereas females either show no change or an increase in cell survival (Gould and Tanapat, 1999; Tanapat et al., 2001; Falconer and Galea, 2003; Pham et al., 2003; Heine et al., 2004; Westenbroek et al., 2004; Shors et al., 2007; Thomas et al., 2007). Thus, these findings suggest that stress differentially affects hippocampal neurogenesis in adulthood but to date no study has examined the effects of stress during adolescence on adult neurogenesis.

Therefore, the aim of this study was to determine the effects of chronic unpredictable restraint stress during adolescence on basal corticosterone levels in adulthood and adult hippocampal neurogenesis in male and female rats. Adolescent male and female rats were exposed to repeated restraint stress every other day for 3 weeks. Hippocampal neurogenesis in the DG (cell proliferation and cell survival) was assessed in adulthood. We hypothesized that exposure to chronic stress during adolescence would reduce neurogenesis in the DG and alter basal corticosterone levels in adulthood in a sex-dependent manner, with the possibility that adult female rats would show greater adverse effects than adult male rats (McCormick et al., 2010).

MATERIALS AND METHODS

Animals

All protocols were in accordance with ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. A total of 32 Sprague–Dawley rats from four dams were used for this study, which were bred in our animal facility. Animals were weaned on postnatal day (PD) 21 and were kept with same-sex siblings for 4 days before they were pair-housed and moved to two different colony rooms (one in which the stressed rats were housed and another in which the nonstressed rats were housed). No more than two offspring per group were drawn from each litter such that in each treatment/sex group at least one rat from each litter was represented. All animals were housed in clear polyurethane bins (48 × 27 × 20 cm) with absorbent bedding and were given Purina rat chow and tap water ad libitum. Rats were maintained on a 12-h light–dark cycle (lights on at 7:30 a.m.).

Procedures

Maternal care observations

Previous research has shown that maternal care, in particular, the amount of time spent licking the offspring can reprogram the HPA axis of male and female offspring determining adult corticosterone baseline levels (Barha et al., 2007; Francis et al., 1999; Liu et al., 1997, 2000). Therefore, we observed maternal care during days 2–8 of the postpartum period using criteria described previously (Brummelte et al., 2006; Pawluski et al., 2006; Barha et al., 2007). Briefly, the amount of time spent licking, nursing and licking, arched back nursing, blanket nursing, passive nursing or off the nest was observed and noted twice a day for 10 min each for each dam. Total time spent licking was calculated for each dam over the course of days.

Restraint stress

Starting on PD30 half of the male ($n = 9$) and half of the female ($n = 7$) rats were exposed to a 1-h restraint stress every other day at unpredictable times for 3 weeks (until PD52 for a total of 12 restraint sessions). During restraint, rats were placed into plastic restraint containers for 60 min in a brightly lit room. The size of the container was adjusted to the growth of the animals, after 1 week for males and 2 weeks for females. Nonstressed controls were left undisturbed with the exception of cage changing twice a week. Body weight was measured on the first day of stress (PD30), half way through the stress period (day 42), and on the last day of restraint stress (PD52) and again in adulthood (PD70).

Blood collection and radioimmunoassay

Blood samples were taken from the tail to assess blood CORT levels of stressed animals at the beginning (t0) and at the end of the restraint session (t60) on the first day of restraint stress (PD30), on the sixth session of restraint stress (PD42), and on the last day of restraint stress (PD52). To minimize the amount of stress for the nonstressed groups, a baseline sample was only taken on PD30. All samples were collected within 3 min of touching the animal's cage or restraint tube, and blood was immediately stored on ice before transferring into a refrigerator.

In adulthood (PD80) another basal blood sample was taken from stressed and nonstressed animals to determine whether chronic restraint stress during adolescence changed basal corticosterone levels in adulthood. Blood samples were centrifuged at 8,000*g* for 8 min 24 h later, and serum was collected and stored at −20°C until further processing.

Serum corticosterone was assayed using a commercial radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) with an intra-assay coefficient of variation less than 7%, an interassay coefficient of variation less than 6%, and a sensitivity of 7.7 ng/ml. All kit reagents and standards were halved and samples were run in duplicates.

Previous work has shown that stage of estrous cycle can influence cell proliferation in female rats with higher levels seen during proestrus (Tanapat et al., 1999). Furthermore, physiological levels of estrone also influence cell proliferation (Barha et al., 2009). Therefore, estradiol and estrone levels were assessed in female rats. Blood was taken at the time of perfusion from the chest cavity. Blood samples were stored overnight at 4°C and centrifuged at 10*g* for 15 min. Briefly, all samples were run in duplicate using commercially available radioimmunoassay kits from Diagnostic Systems Laboratories (Webster, Texas) for estrone and MP Biomedicals (Solon, Ohio) for 17β-estradiol. Intra and interassay coefficient of variation were less than 5% for both kits.

BrdU injection

Adult male and female rats received a single i.p. injection of 5-bromo-2-deoxyuridine (BrdU; 200 mg/kg; Sigma, St. Louis, MO) dissolved in 0.9% saline on PD70 (∼20 days after last restraint stress session). Three weeks later (PD91), all animals were deeply anesthetized with sodium pentobarbital and then perfused with saline followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight and then transferred to 30% sucrose in phosphate-buffered saline (PBS) until sectioning.

Immunohistochemistry

Brains were sliced into 40-μm coronal sections throughout the entire rostral caudal extent of the hippocampus using a vibratome (Leica, VT1000s). Every 10th slice was processed for either Ki67, an endogenous marker of cell proliferation, or for BrdU, an exogenous marker of DNA synthesis that labels progenitor cells and their progeny. In addition, one series of slices were double-stained with fluorescent markers for BrdU and the marker for mature neurons NeuN to allow phenotyping of the stained cells. All immunohistochemistry procedures were performed as previously described (Barha et al., 2009; Epp et al., in press).

Briefly, for Ki67 staining, sections were pretreated with 0.6% H_2O_2 for 30 min, rinsed three times in 0.1 M PBS, and then transferred to a primary antibody solution containing a 1:1,000 rabbit anti-Ki67 monoclonal antibody (Novocastra; Newcastle upon Tyne, UK), 1% normal goat serum, and 0.5% Triton-X in 0.1 M PBS for 16 h. Tissue was rinsed three times again and then incubated in a secondary antibody solution containing 1:1,000 goat antirabbit (Vector; Burlington, ON, Canada) in 0.1 M PBS for 1 h, followed by another rinse (3×) and incubation in an ABC solution (Vector; Burlington, ON, Canada) for 40 min. The sections were rinsed again, developed with diaminobenzidine (DAB; Vector; Burlington, ON, Canada) for 5 min and then mounted on gel-coated glass slides. All sections were lightly counterstained with cresyl violet, dehydrated, and coverslipped with Permount.

BrdU staining was performed similar to Ki67 staining but using Tris-buffered saline (TBS) for rinsing instead of PBS and adding or changing the following steps in the protocol. After initial incubation in H_2O_2 for 30 min and the following rinses, tissue was incubated in 2 N hydrochloric acid at 37°C, followed by one rinse in 0.1 M borate buffer for 10 min and three rinses in TBS. Tissue was then incubated in a solution containing 3% normal horse serum (NHS) and 0.1% Triton-X in TBS for 30 min, before it was transferred to a primary antibody solution containing 1:200 mouse anti-BrdU (Roche; Mississauga, ON, Canada), 3% NHS, and 0.1% Triton-X in TBS for 48 h. The secondary antibody solution contained 1:100 anti-mouse IgG, and tissue was incubated in this solution for 4 h. Incubation time for the ABC solution was 1.5 h.

To assess the phentotype of the BrdU-ir cells, double labeling was performed using BrdU and NeuN, a marker for mature neurons in the brain. Tissue was incubated overnight in 0.1 M TBS containing 3% NHS, 0.1% Triton-X, 1:200 mouse anti-NeuN (Chemicon), and 1:200 rat anti-BrdU (AbD Serotec). Sections were then rinsed 3× in 0.1 M TBS before being incubated overnight in 0.1 M TBS containing 3% normal donkey serum, 0.1% Triton-X, 1:200 donkey anti-mouse Alexa 488 (Invitrogen), and 1:200 donkey anti-rat Cy3 (Jackson). Sections were then rinsed and mounted on glass slides and coverslipped with PVA-DABCO.

Cell counting

Counting was performed by an experimenter blind to the animal's group designation. BrdU- and Ki67-ir cells were counted in every 10th section throughout the entire granule cell layer (GCL), including the subgranular zone (SGZ) using a Nikon microscope at 1,000× magnification. Cells in the hilus were counted separately to account for potential changes in blood–brain barrier permeability. The volume of the DG of

each counted section was measured using an image analysis software (ImageJ, NIH), and the total area was estimated using Cavalieri's principle (Gundersen and Jensen, 1987), that is, by multiplying the total volume measured by the distance between measured sections (400 μm). The total number of BrdU- and Ki67-ir cells was estimated by multiplying the total number counted by 10 as previously described (Kronenberg et al., 2003; Eadie et al., 2005), and total density was calculated by dividing total number of cells by the volume of the DG. The percentage of BrdU/NeuN double-labeled cells was obtained by selecting 25 BrdU cells arbitrarily from at least five sections per brain and determining the percentage of these cells that also expressed NeuN. For this a Nikon epifluorescent microscope (E600) was used at 400× magnification.

Data analyses

Differences in maternal licking received by pups was analyzed using a factorial ANOVA with sex and condition (control, stress exposure during adolescence) as between-subject factors. Body weight was analyzed using a repeated-measure ANOVA with sex (male and female) and condition (control, stress exposure during adolescence) as between-subjects factors and age (PD30, 42, 52, and 70) as the within-subjects factor. Corticosterone levels were analyzed using a repeated-measure ANOVA with sex (male and female) as between-subject factor and day (start, middle, and end of stress) and time (t0 and t60 min) as the within-subject factors. Differences in baseline corticosterone levels in adolescence and adulthood were analyzed using a repeated-measure ANOVA with sex (male and female) and condition (control, stress exposure during adolescence) as between-subject factors and day (PD30 and PD80) as the within-subject factor. Volume of the DG, density of Ki67-ir cells (cell proliferation), and the density of BrdU-ir cells (cell survival) were analyzed using repeated-measure ANOVAs with sex (male and female) and condition (control, stress exposure during adolescence) as between-subject factors and region (GCL + SGZ, hilus) as the within-subject factor. BrdU/NeuN co-labeling was analyzed with a factorial ANOVA with sex and condition as between-subject factors. Post hoc tests used the Newman–Keuls's procedure. All statistical procedures were set at $\alpha = 0.05$.

RESULTS

Offspring in the Different Treatment Groups Did Not Differ in the Amount of Maternal Care Received During the First Week of Life

Results indicate that groups did not differ in the amount of maternal licking received during the first week of life (main effect of sex: $P > 0.87$; main effect of condition: $P > 0.98$; interaction effect: $P > 0.46$).

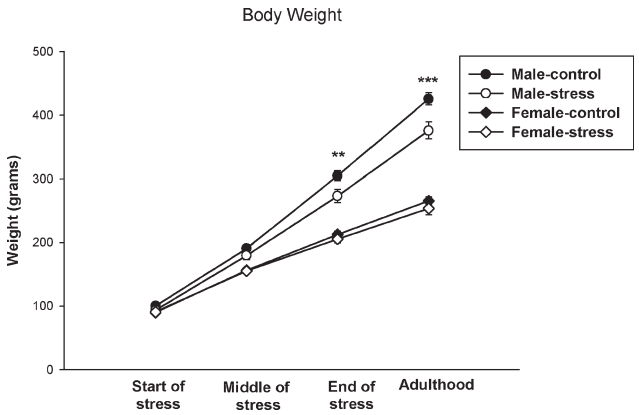

FIGURE 1. Body weight in grams [±standard error of mean (SEM)] for male and female rats at three different time points (PD30, PD42, and PD52) during the 3-week chronic stress exposure period in adolescence and in adulthood (PD70). Female rats exposed to chronic stress during adolescence did not differ from female control rats at any time point during adolescence or during adulthood. Male rats exposed to chronic stress during adolescence did not differ from male control rats at the start (PD30) or the middle (PD42) of the stress exposure period. However, male rats exposed to chronic stress during adolescence had attenuated weight gain at the end of the stress exposure period (PD52) compared to male control rats ($P < 0.01$). This attenuation in body weight gain in males rats exposed to chronic stress during adolescence was still seen in adulthood (PD70) compared to male control rats ($P < 0.001$). Asterisks indicate $^{**}P < 0.01$ versus control; $^{***}P < 0.0001$ versus control.

Exposure to Chronic Stress During Adolescence Leads to an Attenuation in Body Weight Gain in Adolescent and Adult Males Only

A repeated-measure ANOVA on body weight measured at the start (PD30), middle (PD42), and at the end (PD52) of the stress exposure period during adolescence as well as during adulthood (PD70) revealed a significant interaction between day, sex, and condition [$F(3, 84) = 3.21$, $P < 0.05$; see Fig. 1]. Post hoc tests revealed that exposure to stress during adolescence attenuated weight gain only at the end of the stress period (PD52) in males compared to controls ($P < 0.01$). This attenuation in body weight gain in male rats exposed to chronic stress during adolescence was still seen in adulthood compared to controls ($P < 0.001$). Male rats exposed to chronic stress did not differ from controls in body weight at the start or the middle of the stress period (both P's > 0.32). Female rats exposed to chronic restraint stress did not significantly differ from controls in body weight at any time point (all P's > 0.26).

Adolescent Males Show a Greater Corticosterone Response to Restraint Stress Compared to Adolescent Females

A repeated-measure ANOVA on total serum corticosterone levels at 0 and 60 min after the onset of restraint stress on the first, middle, and last day of the stress period revealed a signifi-

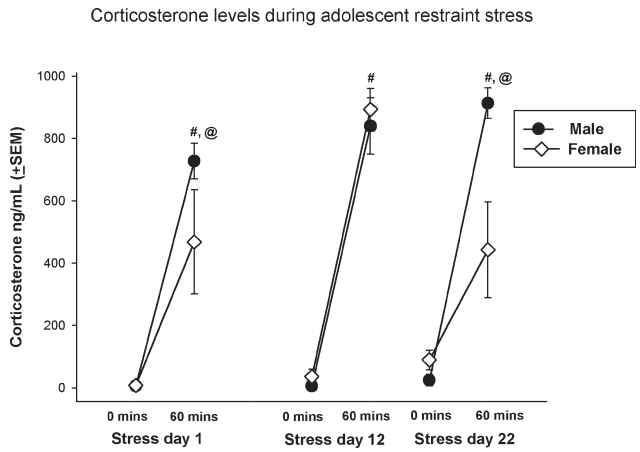

FIGURE 2. Total serum corticosterone (ng/ml) levels (±SEM) for adolescent males and females exposed to 3 weeks of chronic restraint stress at baseline and 60 min after the onset of restraint stress on day 1, 12, and 22 of the stress exposure period. Adolescent males and females did not significantly differ in baseline levels of corticosterone at any time point during the stress exposure period (all *P*'s > 0.80). Both adolescent males and females had higher levels of serum corticosterone 60 min after restraint stress compared to baseline throughout the stress period (all *P*'s < 0.01). Males responded with higher corticosterone levels after 60 min of restraint compared to females on the first day and the last day (day 22) of the stress exposure period (both *P*'s < 0.05) but not on the middle day (day 12) of the stress exposure period (*P* > 0.60). # indicates significantly different from baseline; @ indicates significantly different between males and females.

cant interaction between sex, time, and day [$F(2, 28) = 4.25$, $P < 0.024$; see Fig. 2]. Post hoc tests revealed that male and female rats did not significantly differ in baseline levels of corticosterone on the first day ($P > 0.99$), middle day ($P > 0.99$), or the last day ($P > 0.84$) of stress exposure. Both males and females had higher levels of serum CORT 60 min after restraint stress on the first day (both P's < 0.001), middle day (both P's < 0.001), and the last day (both P's < 0.01) of stress compared to baseline. However, males responded with higher levels of corticosterone after 60 min of restraint compared to females on the first day ($P < 0.02$) and the last day ($P < 0.01$) but not on the middle day ($P > 0.62$) of stress exposure. There were also significant interactions between sex and day [$F(2, 28) = 4.00$, $P < 0.03$], sex and time [$F(1, 14) = 8.12$, $P < 0.01$], and time and day [$F(2, 28) = 4.29$, $P < 0.02$]. There were also significant main effects of time [$F(1, 14) = 231.44$, $P < 0.0001$], day [$F(2, 28) = 5.04$, $P < 0.01$], and a tendency for an effect of sex [$F(1, 14) = 3.45$, $P = 0.08$].

Exposure to Chronic Stress During Adolescence Increases Baseline Corticosterone in Adult Females, But Not Adult Males

A repeated-measure ANOVA on total serum corticosterone levels at baseline taken during adolescence (PD30) and adulthood (PD80) revealed a significant interaction between day, sex, and condition [$F(1, 24) = 4.08$, $P < 0.05$; see Fig. 3]. Post hoc tests revealed that groups did not differ in baseline corticosterone levels during adolescence (all P's > 0.92). On the other hand, adult female rats exposed to restraint stress in adolescence had higher baseline corticosterone levels in adulthood than did female control rats ($P < 0.001$), but this difference was not observed in male rats ($P > 0.83$). Baseline corticosterone levels were higher in adulthood than in adolescence in both male and female rats regardless of condition ($P < 0.014$ for males and $P < 0.001$ for females). Baseline corticosterone levels in female rats, regardless of condition, were higher than in male rats regardless of condition in adulthood (all P's > 0.05). Significant two-way interactions {day and sex [$F(1, 24) = 18.98$, $P < 0.001$] and sex and condition [$F(1, 24) = 4.30$, $P < 0.05$]} and main effects {age [$F(1, 24) = 74.70$, $P < 0.001$]; sex [$F(1, 24) = 19.10$, $P < 0.001$]; and day [$F(1, 24) = 74.77$, $P < 0.0001$]} were also found.

Previous studies have shown that corticosterone levels are elevated during the proestrous phase of the estrous cycle (Carey et al., 1995; Atkinson and Waddell, 1997); therefore, serum samples collected in adulthood (PD80) for corticosterone assessment were also analyzed for 17β-estradiol levels. Results indicate that three females in the stress group were in proestrus,

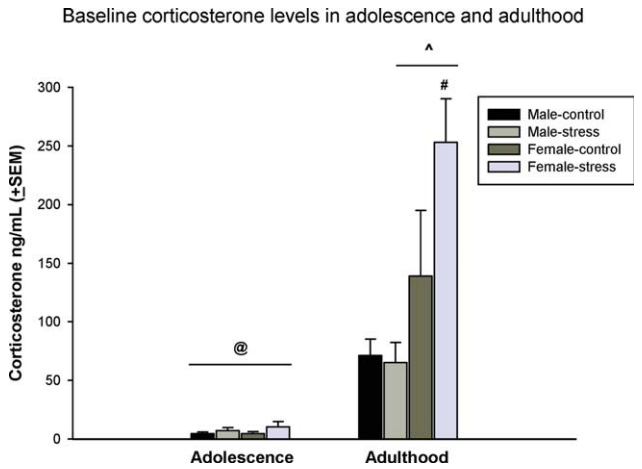

FIGURE 3. Total serum corticosterone (ng/ml) levels (±SEM) at baseline in adolescence (PD30) and adulthood (PD80) for male and female rats exposed to chronic restraint stress in adolescence and control male and female rats. Male and female rats exposed to chronic restraint stress in adolescence and control rats did not differ in baseline corticosterone levels during adolescence (all *P*'s > 0.92). Baseline corticosterone levels were higher in adulthood than in adolescence in male and female rats regardless of condition (both *P*'s < 0.01). In adulthood, female rats had significantly higher baseline corticosterone levels than male rats regardless of condition (*P*'s > 0.05). In adulthood, female rats exposed to chronic restraint stress in adolescence had significantly higher baseline corticosterone levels than female control rats not exposed to chronic restraint stress in adolescence (*P* < 0.001), but male groups did not differ (*P* > 0.83). @ indicates adult male and female rats significantly different from adolescent male and female rats regardless of condition; ^ indicates adult female rats significantly different from adult male rats regardless of condition; #indicates adult female rats exposed to chronic stress during adolescence significantly different from adult control females. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1.

Total Volume of the Granule Cell Layer and the Hilus and Percentage of BrdU-ir Cells Co-labeled with NeuN (±SEM) in Adulthood of Male and Female Rats Exposed to Chronic Stress in Adolescence and Adult Control Female Rats Not Exposed to Chronic Stress in Adolescence

Group	GCL (±SEM)	Hilus (±SEM)	% of BrdU/NeuN
Male-control	2.88 ± 0.08	6.38 ± 0.44	84% ± 0.08
Male-stress	2.67 ± 0.08	5.85 ± 0.21	82% ± 0.04
Female-control	2.61 ± 0.11	6.02 ± 0.26	81% ± 0.02
Female-stress	2.61 ± 0.08	5.69 ± 0.39	80% ± 0.04

whereas two females in the control group were in proestrous (data not shown). Importantly, removing the data from the females in proestrus did not alter the findings that adult female rats exposed to restraint stress in adolescence had higher baseline corticosterone levels in adulthood than did female control rats ($P < 0.03$). Furthermore, 17β-estradiol levels did not differ between stress and control females [$F(1, 10) = 0.05$, $P > 0.80$], and baseline corticosterone levels did not differ between proestrus females in the stress group and control group [$F(1, 3) = 0.16$, $P > 0.70$]. Furthermore, baseline corticosterone in adulthood did not correlate with 17β-estradiol levels ($r = 0.42$, $P > 0.16$).

Exposure to Chronic Stress During Adolescence Does Not Alter DG Volume in Adult Female and Male Rats

Results indicate that groups did not differ in the volume of the granule cell layer or the hilus (main effect of sex: $P > 0.29$; main effect of condition: $P > 0.18$; interaction effect: $P > 0.59$; Table 1). As expected, there was a significant main effect of region with greater hilar volumes than GCL volumes [$F(1, 28) = 486.79$, $P < 0.0001$]. No other significant interactions were found (all P's > 0.28).

Exposure to Chronic Stress During Adolescence Decreases Cell Proliferation and Cell Survival in Adult Female Rats But Tends to Increase Cell Survival in Adult Male Rats

Previous work has shown that high levels of 17β-estradiol seen during the proestrous stage of the estrus cycle increases cell proliferation compared to other stages of the estrus cycle (Tanapat et al., 1999). Furthermore, physiological levels of estrone also influence cell proliferation (Barha et al., 2009). Therefore, 17β-estradiol and estrone levels were measured in the current experiment to assess the stage of estrus cycle each female rat was in at the time of perfusion (assessment of cell proliferation). None of the female rats were in proestrus when cell proliferation was assessed (estrone and estradiol, data not shown). Furthermore, 17β-estradiol levels did not correlate with cell proliferation levels ($r = 0.15$, $P > 0.65$), nor did estrone levels ($r = 0.02$, $P > 0.96$).

A repeated-measure ANOVA on density of Ki67-ir cells in the GCL and hilus of the DG revealed a significant interaction between region, sex, and condition [$F(1, 26) = 4.55$, $P < 0.05$; see Fig. 4a]. Post hoc tests show that stressed female rats

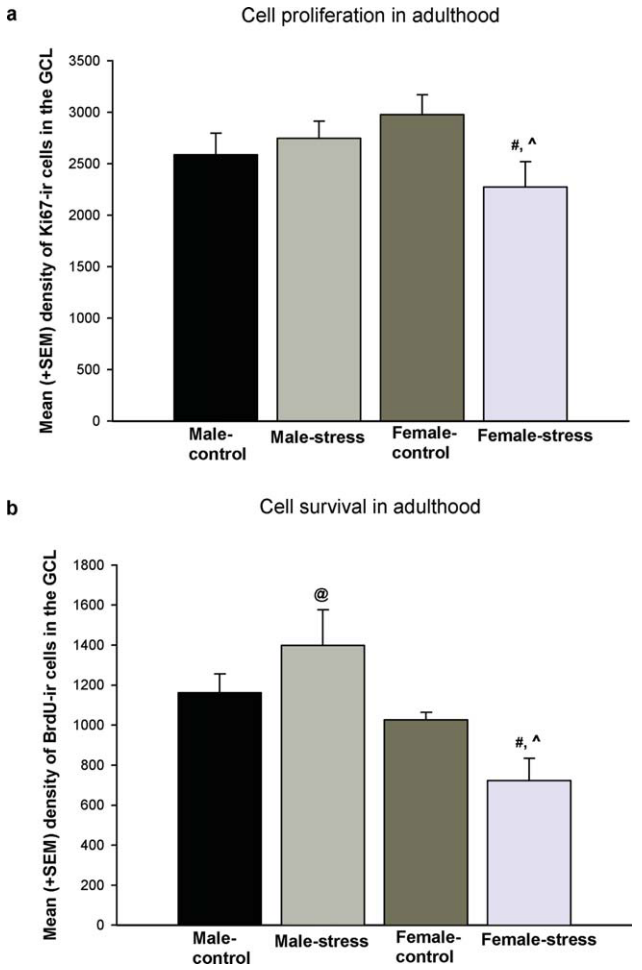

FIGURE 4. (a) Mean density (+SEM) of Ki67-ir cells in the GCL of adult male and female rats. Adult female rats exposed to chronic restraint stress in adolescence had a significantly lower density of Ki67-ir cells compared to female control rats ($P < 0.001$) and tended to have a lower density of Ki67-ir cells compared to adult male rats exposed to chronic restraint stress in adolescence ($P = 0.07$). # indicates significantly different from control group of the same sex; ^ indicates tendency to be differ from the stress group of the other sex. (b) Mean density (+SEM) of BrdU-ir cells in the GCL of adult male and female rats. Adult female rats exposed to chronic restraint stress in adolescence had a significantly lower density of BrdU-ir cells compared to female control rats ($P < 0.05$) and male rats exposed to chronic restraint stress in adolescence ($P < 0.001$). Adult male rats exposed to chronic restraint stress in adolescence tended to have a higher density of BrdU-ir cells compared to male control rats ($P = 0.06$). # indicates significantly different from control group of the same sex; # indicates significantly different from the stress group of the other sex; @ indicates tendency to differ from control group of the same sex. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

had a lower density of Ki67-ir cells in the GCL compared to control female rats ($P < 0.001$) and tended to have lower density of Ki67-ir cells compared to stressed male rats ($P = 0.07$). There were no other significant differences between groups in the density of Ki67-ir cells in the GCL or the hilus (all P's > 0.14). A significant interaction between sex and condition [$F(1, 26) = 4.08$, $P < 0.05$] and a significant main effect of region [$F(1, 26) = 622.67$, $P < 0.0001$] were also seen.

A repeated-measure ANOVA on density of BrdU-ir cells in the GCL and hilus revealed a significant interaction between region, sex, and condition [$F(1, 19) = 4.19$, $P < 0.05$; see Fig. 4b]. Post hoc tests show that stressed female rats had a lower density of BrdU-ir cells in the GCL compared to control female rats ($P < 0.05$) and stressed male rats ($P < 0.001$). Control female rats did not differ from control male rats ($P > 0.30$). Control male rats tended to have a lower density of BrdU-ir cells in the GCL compared to stressed male rats ($P = 0.06$). Groups did not differ in density of BrdU-ir cells in the hilus (all P's > 0.77). There were also significant two-way interactions {sex and condition [$F(1, 19) = 5.34$, $P < 0.05$] and region and sex [$F(1, 19) = 11.35$, $P < 0.01$]} and significant main effects {sex [$F(1, 19) = 10.32$, $P < 0.01$] and region [$F(1, 19) = 301.90$, $P < 0.0001$]}.

Exposure to Chronic Stress During Adolescence Does Not Alter the Percentage of New Neurons in Adult Female and Male Rats

Results indicate that groups did not differ in the percentage of BrdU-ir cells that were colabeled with NeuN in the GCL (main effect of sex: $P > 0.43$; main effect of condition: $P > 0.63$; interaction effect: $P > 0.87$; Table 1).

DISCUSSION

The results from this study demonstrate that exposure to chronic, intermittent stress during adolescence alters neurogenesis in DG of the hippocampus, and basal corticosterone levels in adulthood in female, but not male, rats. In addition, male, but not female, rats exposed to restraint stress in adolescence had attenuated body weight gain as seen at the end of the stress period in adolescence and in adulthood. Although males and females responded with elevated corticosterone levels to the stressor throughout the stress period, males responded with higher corticosterone levels at 60 min than did female rats on the first and last day of the stress period. In adulthood only female rats exposed to stress during adolescence had higher baseline levels of corticosterone compared to control female rats. Although exposure to chronic intermittent stress during adolescence did not alter the volume of the DG in either sex, cell proliferation and cell survival/neurogenesis were decreased in adult females compared to controls. Taken together, these results indicate that despite males having a greater response to the stressor in adolescence, females show long-term alterations in hippocampal neurogenesis in adulthood. To our knowledge this is the first demonstration of how stress during adolescence impacts adult neurogenesis in the DG of male and female rats.

Exposure to Chronic Stress During Adolescence Induces Different Corticosterone Responses in Males and Females and Attenuates Body Weight Gain in Males Only

Male rats exposed to chronic, intermittent stress for 3 weeks during adolescence had lower body weight at the end of the stress period compared to control male rats that were not exposed to stress. Importantly, this effect on body weight in males was maintained into adulthood. Female rats exposed to chronic stress during adolescence did not differ in body weight from control females at any time point. Our results are consistent with previous studies showing an attenuated body weight gain after exposure to chronic stress during adolescence in males but not females (McCormick et al., 2005; McCormick and Ibrahim, 2007), an effect lasting into adulthood (McCormick et al., 2004).

Consistent with previous work, in this study, both males and females responded to the restraint stress with elevated corticosterone levels 60 min after the onset of stress (Brummelte et al., 2006; Barha et al., 2007) and neither sex completely habituated to the stressor throughout the 3-week stress period (Romeo et al., 2006b). Specifically, the female rats exposed to stress during adolescence may have been starting to habituate to the stressor as they showed lower levels of corticosterone at 60 min after the onset of stress on the last day of the stress period compared to male rats. Furthermore, their CORT profile in response to stress after 60 min did not increase from day 1 to day 22 of the repeated restraint testing as it did in males during adolescence. This is in partial contrast to the data from male rats that show that the corticosterone response to stress does not as readily habituate in adolescence as it does in response to stress in adulthood (Romeo et al., 2006a). On the other hand, adolescent and adult female rats have been previously shown to habituate to chronic stress (Doremus-Fitzwater et al., 2009), which was partially consistent with our observations. It is important to note that in our study we only looked at one time point, 60 min after the onset of stress, and it is possible that had we used other time points we may have seen different habituation profiles (see below). Interestingly, male rats showed a greater response to the restraint stress than did females on the first and last day of stress testing. This difference between males and females in HPA response to the stressor may in part be due to sex differences in pubertal development of the HPA axis (McCormick and Mathews, 2007) as sex differences in adrenal weight emerge after 50 days of age (Sencar-Cupovic and Milkovic, 1976). Furthermore, although it appears that adolescent and adult female rats can show higher peak corticosterone responses to stress than males (McCormick et al., 2007), females rats at both ages also show faster return to baseline (Romeo et al., 2004a,b). Therefore, it is possible that in this study the lower corticosterone levels seen in females at 60 min after the onset of stress on the first and last days of

the stress period compared to males may be due to this quicker ability of female rats to return to baseline levels. It is possible that we may not have seen our particular pattern of results if we had sampled blood at a time point earlier than 60 min after the onset of stress. Thus, these results showing a greater CORT response to the stressor in adolescent males compared to adolescent females may be due to a number of factors such as sex differences in HPA development, timepoint after stress initiation to examine CORT, or salience of the stressor.

Interestingly, exposure to chronic intermittent restraint stress during adolescence increased baseline corticosterone levels in adulthood in female rats; however, it did not influence baseline corticosterone levels in adult male rats. These findings are not completely consistent with earlier studies conducted by Mathews et al. (2008) and McCormick et al. (2008), who found that baseline corticosterone levels in adulthood were not influenced by adolescent stress in males or females. However, stress paradigms differed between the studies as Mathews et al. (2008) and McCormick et al. (2008) exposed males and females to chronic social stress defined as 1 h of social isolation followed by exposure to a new cage mate each day and occurred on days 30–45 of age, whereas this study exposed males and females to 1 h of restraint stress every other day at unpredictable times from 30 to 52 days of age. The finding in this study that females exposed to restraint stress in adolescence have higher baseline corticosterone levels in adulthood compared to control females was not due to estrous cycle stage. It may be possible that exposure to stress in adolescence may have permanently altered glucocorticoid receptor (GR) levels and distribution in female rats leading to heightened baseline levels seen in adulthood (see below for further discussion).

Exposure to Chronic Stress During Adolescence Alters Hippocampal Plasticity Differently in Males and Females

Exposure to chronic intermittent restraint stress during adolescence did not alter DG volume in male or female adult rats. This is somewhat inconsistent with a previous study that found exposure to chronic unpredictable stress during adolescence resulted in decreased volume of CA1, CA3, and DG regions of the hippocampus in male rats. This reduction in hippocampal volume was seen 3 weeks after the cessation of the stress, indicating that these effects of stress on the developing adolescent brain are delayed (Isgor et al., 2004). However, it is important to note that the control rats in that study were handled daily. Although adolescent handling has no effect on hippocampal cell survival in male gerbils (Schaefers et al., 2009), postnatal handling, a mild form of enrichment, can alter hippocampus structure and volume (O'Donnell et al., 1994; Meaney et al., 2000; Lehmann et al., 2002; Lemaire et al., 2006) and can change the activation of stress response circuits (Abraham and Kovacs, 2000). Thus, it is conceivable that daily handling during adolescence or different types of stressors might influence hippocampal volume in adulthood. Interestingly, exposure to chronic stress during adulthood can lead to a reduction in hippocampal volume if the stress is severe and prolonged enough [(Pham et al., 2003; for review, see Sapolsky (2000)]. For example, using structural magnetic resonance imaging, chronic restraint stress decreases hippocampal volume by 3% in adult male rats (Lee et al., 2009). Thus, the ability of chronic stress to decrease hippocampal volume is dependent on a number of variables including developmental time of exposure, amount of stress and stressor type.

Exposure to chronic intermittent restraint stress during adolescence influenced hippocampal neurogenesis differently in adult male and female rats. Specifically, there was a decrease in cell proliferation and cell survival/neurogenesis in adult female rats and a slight increase in cell survival in adult male rats. Cell proliferation was not affected by chronic adolescent stress in male rats. To our knowledge, this is the first study to investigate the effects of chronic stress during adolescence on neurogenesis in the DG of adult male and female rats. Previous work has shown that chronic stress during adolescence slightly increases new cells in the DG after short-term survival (5 days) in young males (Toth et al., 2008), consistent with our present results. Also consistent with our results, chronic social stress during adolescence decreased new cell survival in the DG after short-term survival (4 days) in adolescent female rats (McCormick et al., in press). Furthermore, stress from maternal separation on PD3 increased hippocampal neurogenesis in males but decreased hippocampal neurogenesis in females on PD21 (Oomen et al., 2009). Thus, these findings coupled with our own suggest that adolescent stress differentially influences neurogenesis in the DG of male and female rats with females showing a decrease and males showing a slight increase in adult neurogenesis.

Stress-induced changes in glucocorticoid levels can have a direct effect on neurogenesis as both GRs and mineralocorticoid receptors (MR) are found on granule cells in the DG (Reul and de Kloet, 1985), and 10–15% of the progenitor cells express both these receptors (Cameron et al., 1993; Garcia et al., 2004). Importantly, expression of GR and MR in the hippocampus reaches adult levels by PD21 (for review see McCormick et al. (in press). However, exposure to chronic stress during adolescence leads to a decrease in expression of GR and MR in the adult hippocampus (Uys et al., 2006; Schmidt et al., 2007; Sterlemann et al., 2008), possibly reflecting a long-term impairment of the negative feedback system of the HPA. Thus, the sex-dependent effects of chronic adolescent stress on adult neurogenesis might be mediated through changes in GR and MR expression levels or through stress-induced long-term changes in HPA axis function.

Age Matters in Neural and Behavioral Response to Stress: Adolescent Versus Adult Stress

In this study our findings on adult neurogenesis after chronic adolescence stress are in stark contrast to the effects that chronic stress in adulthood has on adult neurogenesis. Exposure to acute or chronic stress in adulthood decreases hippocampal cell proliferation and neurogenesis in adult males but has variable effects on adult hippocampal neurogenesis and proliferation in female rats (Tanapat et al., 2001; Holmes and Galea, 2002; Falconer

and Galea, 2003; Pham et al., 2003; Joels et al., 2004; Toth et al., 2008). For example, chronic footshock stress in adulthood decreased cell survival in males, but increased cell survival in females, when BrdU was given early during the stress regimen (Westenbroek et al., 2004). However in our study, stress during adolescence decreased hippocampal neurogenesis in adult females and increased hippocampal neurogenesis in adult males. Combined, these results suggest that stress alters neurogenesis in a sex- and age-dependent manner, with different patterns of results seen in adolescent and adult rats.

Adolescent and adult rats also differ in the HPA response to stress. Specifically, adolescent rats respond to an acute stressor with higher and more prolonged corticosterone release than adult rats (Romeo et al., 2004a,b, 2006b; Cruz et al., 2008). Adult male rats maintain elevated corticosterone levels for longer early on during the stress compared to adolescent male rats despite the fact that adolescent male rats do not habituate to chronic stress as adult male rats do (Romeo et al., 2006a). Perhaps surprisingly, adolescent and adult rats do not differ in GR mRNA levels in the hippocampus after exposure to chronic stress (Romeo et al., 2008). Interestingly, exposure to chronic mild stress decreases levels of brain-derived neurotrophic factor and levels of AMPA receptor GluR1 subunit in the hippocampus of adult male rats, but not in younger male rats (Toth et al., 2008). These findings suggest that stress exerts differential effects with experience during adolescence or adulthood.

Sex Matters in Neural and Behavioral Response to Stress

The results of this study add to the growing literature on sex differences in biological responses to stress, which may in part be due to differences between males and females in HPA activation after stress (Bale, 2006; Kajantie and Phillips, 2006). For example, adult male rats show suppressed cell proliferation and cell survival in response to acute and chronic stress (Tanapat et al., 2001; Falconer and Galea, 2003; Pham et al., 2003; Heine et al., 2004; Westenbroek et al., 2004; Shors et al., 2007; Thomas et al., 2007). However, the effects of stress on adult neurogenesis in females are more complex and not as consistent. Some studies show a stress-induced decrease in neurogenesis in adult female rats (Kuipers et al., 2006), whereas others show no significant effect or an increase in neurogenesis in adult female rats (Falconer and Galea, 2003; Westenbroek et al., 2004; Shors et al., 2007). The effects of stress on neurogenesis in females are dependent on many factors including hormonal status, length of exposure to stressor, and controllability of the stressor (Tanapat et al., 2001; Falconer and Galea, 2003; Shors et al., 2007; Thomas et al., 2007). Interestingly, recent data from our laboratory show that both male and female rats show decreased neurogenesis after chronic corticosterone exposure in adulthood (Brummelte and Galea, 2010), suggesting that high levels of corticosterone can reduce neurogenesis in both males and females.

In addition to sex differences in the neurogenic response to chronic stress, adult females show different neurotransmitter changes in the brain and cognitive performance after stress compared to males (Luine, 2002; Bowman et al., 2003, 2009; Dalla et al., 2008). For example, following chronic stress, adult female rats show enhanced performance when male rats show impaired spatial performance (Bowman et al., 2001, 2002; Beck and Luine, 2002; Conrad et al., 2003; Kitraki et al., 2004). In contrast, following acute stress, adult male rats show facilitated classical conditioning but adult female rats show impaired classical conditioning (Wood and Shors, 1998). Interestingly, males and females also show different neurtransmitter activity in the frontal cortex and hippocampus after chronic stress (Bowman et al., 2003). For instance, males, but not females, showed a reduction in dopaminergic metabolites in the frontal cortex after 21 days of chronic restraint stress. Therefore, the behavioral and neural effects of stress are dependent on the sex of the subject.

CONCLUSIONS

Stressful experiences that occur during the adolescent period have a profound impact on the structure and function of the hippocampus, and many effects are seen well into adulthood. The results of this study indicate that exposure to chronic, intermittent restraint stress during adolescence alters the neurogenic and basal hormonal status of adults in a sex-specific manner. Female rats were adversely affected showing a reduction in cell proliferation and cell survival and elevated basal corticosterone levels in adulthood. Although male rats were more responsive to the chronic stress as adolescents, as adults they showed a slight increase in cell survival and no affect of basal corticosterone levels, suggesting a different possibly protective or compensatory mechanism to deal with adolescent stress in males compared to females. These results are particularly interesting in light of clinical reports that a higher prevalence of stress-related psychological illnesses are found in women. Therefore, it may be the case that females are at a greater risk for long-term, permanent effects of early-life adversity. Further research is required to elucidate the specific neurobiological mechanisms at play in males and females.

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