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Perinatal Stress Effects on Later Anxiety and Hormone Secretion in Male Mandarin Voles

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The estradiol (E_2), estrogen receptor-α (ERα), testosterone (T), and androgen receptor (AR) can contribute to anxiety, but whether they are associated with the reversion of prenatal adverse outcomes remains unclear. Here, we tested the interactive effects of prenatal maternal restraint stress and early postnatal short-term maternal separation on adult male mandarin vole (*Microtus mandarinus*) behavior and changes in E_2, T, and their receptors. The results showed that PS adult males (PS/NH) exhibited an increase in anxiety-like behavior in open-field and elevated plus-maze tests than the other 3 groups, including adult male offspring controls (PC/NH), adult male offspring controls with short-term maternal separation (PC/H), and PS adult males with short-term maternal separation (PS/H). The increase in anxiety-like behavior was associated with significantly lower E_2 and T serum levels, had significantly more ERα immunoreactive neurons (ERα-IRs) in some brain regions, as well as significantly fewer AR immunoreactive neurons (AR-IRs) in some brain regions than the other 3 groups. We found it interesting that the PC/H and PS/H were similar to the PC/NH in that they did not produce anxiety-like behavior. However, early postnatal short-term maternal separation reversed prenatally induced changes in E_2 and T serum levels and altered ERα-IRs and AR-IRs in the brain. These data suggest that changes in anxious adults may be governed by early environmental factors and their interactions because changes in E_2 and T serum levels and the distribution of ERα and AR in the brain result in behavioral changes related to less anxiety into adulthood.

Keywords: androgen receptor, estrogen receptor-α (ERα), prenatal stress, estrogen (E_2), testosterone (T)

Anxiety and depression are highly prevalent chronic and potentially life-threatening neurological disorders (Nestler et al., 2002). The hypothalamic–pituitary–adrenal (HPA) axis is a highly plastic and sensitive system, and dysregulation of the HPA axis causes anxiety (Van den Bergh, Van Calster, Smits, Van Huffel, & Lagae, 2008). The steroid hormones estradiol (E_2) and testosterone (T) affect the HPA axis, and E_2 and T respectively increase and attenuate adrenocorticotropic hormone (ACTH) and corticosterone secretion (Franklin, Saab, & Mansuy, 2012). Therefore, abnormal secretion of estrogen and androgens can also contribute to mood disorders (Morrison, Brinton, Schmidt, & Gore, 2006). For example, several lines of evidence suggest that anxiety disorders are most frequent during times when estrogen levels are low and may be relieved by estrogen treatment (Best, Rees, Barlow, & Cowen, 1992; Sichel, Cohen, Robertson, Ruttenberg, & Rosenbaum, 1995; Arpels, 1996; Gregoire, Kumar, Everitt, & Studd, 1996). Lower levels of testosterone and testosterone levels that decreased more slowly over the course of a day were related to higher levels of anxiety/depression symptoms and attention problems in adolescent boys (Granger et al., 2003). Low testosterone levels in hypogonadal and aging men have been associated with diagnosed depression (Eskelinen, Vahlberg, Isoaho, Kivelä, & Irjala, 2007). It has also been shown that T treatment in mice can decrease symptoms of anxiety (Zuloaga, Morris, Jordan, & Breedlove, 2008).

Prenatal stress (PS) as a result of maternal stress can result in an increased risk of anxiety and depression in offspring (Howerton & Bale, 2012), which can lead to changes in E_2 and T levels and influence the HPA axis and regulate the activity and behavior of male offspring (Kapoor & Matthews, 2011; Tchernitchin, Tchernitchin, Mena, Unda, & Soto, 1999). The effect of estrogen on anxiety may be explained by estrogen causing changes in the estrogen receptor-α (ERα) in particular brain regions (Newhouse et al., 2010; Fedotova, 2013). The expression of ERα is found in abundance in the medial amygdala, the bed nucleus of stria terminalis (BNST), and the preoptic area (Krezel, Dupont, Krust, Chambon, & Chapman, 2001). ERα activation has been shown to increase fear and anxiety in male mice and rats (Morgan & Pfaff, 2001; Patisaul & Bateman, 2008). However, the relationship between the distribution of ERα and anxiety disorders in other animals remains unreported except in mice and rats.

Male rat offspring born to mothers exposed to restraint stress during the last week of pregnancy have decreased plasma T levels, elevated basal ACTH levels, and blunted responses to stress (Richardson, Zorrilla, Mandyam, & Rivier, 2006). Thus, evidence suggesting a role for T in mediating the effects of PS on HPA-axis

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activity and behavior in male offspring is accumulating. Androgens play an important role in rodents by acting on androgen receptors (ARs) in key brain areas, such as the medial preoptic area (mPOA) and the medial amygdaloid nucleus (MeA; Bingham, Williamson, & Viau, 2006; Williamson & Viau, 2007). Under certain stressors, androgens may affect anxiety-related behavior by acting through ARs in brain regions such as the BNST, MeA, and hypothalamic paraventricular nucleus (PVN; Edinger & Frye, 2006).

The postnatal environment can also have a considerable impact on the development of an organism. To assess the effects of the postnatal environment on behavior and physiology, numerous studies have explored the effects of early separation from the mother on development of the offspring. Separation periods can range from a single separation during the preweaning period (Ellenbroek, van den Kroonenberg, & Cools, 1998) to repeated daily separations (15 min to 5 hr; Brake, Zhang, Diorio, Meaney, & Gratton, 2004; Meaney et al., 1991). In addition, separations can encompass almost the entire preweaning period (such as in artificial rearing; see Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001; Lovic & Fleming, 2004). On the basis of these temporal parameters, maternal separation studies have found multiple and opposing effects on behavior and physiology. Elevated indices of anxiety have been exhibited as a result of long periods of daily separation (Ellenbroek et al., 1998; Huot et al., 2002). In contrast, short periods of separation, or handling regimens have exhibited reduced separation anxiety responses in pups (Lehmann, Stöhr, & Feldon, 2000).

The mandarin vole (*Microtus mandarinus*) is a monogamous rodent that engages in pair bonding, affiliation, and biparental care (Guo et al., 2011; Tai, Wang, & Zhou, 2001). Social-bonding and pair-bonding formation are critical components of mental health (Young, Gobrogge, Liu, & Wang, 2011), and mandarin voles have emerged as a model for the study of social–emotional behaviors such as those associated with anxiety (He, 2014) and depression (He, Tai, Zhang, & Zhang, 2012). Previous experiments by our group have shown that PS (e.g., exposure to ethanol) increases anxiety and decreases central AR expression in adult mandarin voles (He, 2014). E_2, ERα, T, and AR can contribute to anxiety, but whether they are associated with the reversion of prenatal adverse outcomes is unclear. Therefore, the aims of the present study were to (a) determine whether a correlation exists between prenatal maternal restraint stress and the distribution of ERα and ARs in brain regions associated with anxiety-like behavior; and (b) investigate whether early postnatal maternal separation can reverse these behaviors, and whether E_2 and T serum levels and the distribution of ERα-IRs and AR-IRs in the brain change accordingly.

Method

Animals

All experimental animals included were female and male mandarin voles whom we obtained from an outbred colony and fed at the College of Biotechnology, Xi'an University, Xi'an, PRC. Mandarin voles were captured from Lingbao voles in Henan, China, 1997. Adult females ($N = 40$, 30–36 g, 90 days old) were individually housed in clear plastic cages (30 × 20 × 15 cm) and were maintained in a 14:10-hr light/dark cycle at 24–26 °C. Hardwood shavings and cotton were provided as substrate and bedding. Rabbit chow (Laboratory Animals Center, Xi'an Medical University, Xi'an, China), carrot, and malt were provided ad libitum. All methods were approved by the Institutional Animal Care and Use Committee, Xi'an University of Arts and Science. To avoid exogenous and endogenous environmental factors that might have induced differences between animals (e.g., infection, age, sisterhood), we captured new mandarin voles from Lingbao each year and chose healthy animals of similar mass. These new voles are paired with laboratory voles, and their offspring supplement our laboratory population to avoid inbreeding.

Monogamous female voles do not display spontaneous ovarian activity or ovulation in nonestrus (Sawrey & Dewsbury, 1985) and females are not receptive to males except when they are in estrus. However, estrus can be induced in females through estrogen administration alone (Carter, Witt, Auksi, & Casten, 1987). Once fertilization occurs, continued male presence significantly increases the probability that the pregnancy will be carried to term. Therefore, female mandarin voles were brought into estrus with estradiol benzoate (0.75 μg/g, 24 hr before testing) and progesterone (0.015 mg/g, 4–6 hr before testing) and we monitored the estrus state of females using vaginal smears. Each female in estrus was paired with an adult male whose bilateral or unilateral testes had descended ($N = 40$; total 40 pairs). Pregnancy began when two ejaculations were observed (Day 0 of pregnancy; Ward, Ward, Denning, French, & Hendricks, 2002).

Prenatal Stress Treatment

The gestation period in mandarin voles is about 21 days, as it is in rats and mice. The fetal and neonatal development of the testis of a sexually undifferentiated gonad involves a succession of events allowing the development of each testicular cell type (Olaso & Habert, 2000). In the rat, Sertoli cells differentiate and surround the germ cells to form the seminiferous cords from 13.5 to 14.5 days postconception (dpc), and then they proliferate until 3 weeks after birth (Pelliniemi, Frojdman, & Paranko, 1993). Therefore, we speculate that testicular development occurs in the fetal male vole from 13.5 to 14.5 days postconception. In the present experiment, the period of maternal stress began after the development of the testes. Forty pregnant female mandarin voles were randomly assigned either to a PS or a PC group. Pregnant female voles in the PS group ($N = 20$) were subjected to restraint stress during the last 2 weeks of pregnancy. The restraint-stress treatment involved removing the female from its home cage and placing it in a closed, narrow, transparent plastic tube (11 cm in length × 6 cm in diameter) for 30 min, 3 times a week, at different times in the morning. The control group ($N = 20$) was left undisturbed in their cages. One to five pups were produced in each litter, and the sex ratio was 1.87 females to 1 male (He, Tai, et al., 2012; Tai, Wang, & Zhao, 1999). To exclude litter effects, only one male pup was chosen from each litter: The 20 male pups from the treatment group were denoted as PS, and the 20 male pups from the control group as prenatal control (PC). The extra pups in each litter were used in other experiments conducted by our group. Thus, these offspring were all from the dam's first litter in the present experiment, and dams were housed with sires throughout the prenatal and preweaning periods.

Early Postnatal Short-Term Maternal Separation

The PS group was divided into two groups: PS with postnatal short-term maternal separation (i.e., handling; PS/H, $N = 10$) and PS without postnatal short-term maternal separation (i.e., no handling; PS/NH, $N = 10$). The PC group was divided into two groups: PC with postnatal short-term maternal separation (PC/H, $N = 10$) and PC without postnatal short-term maternal separation (PC/NH, $N = 10$). All groups were housed in the same animal cages, and all pups were kept together with their mothers. Postnatal short-term maternal separation was performed daily from the 1st to the 14th postnatal day. Separated litters were removed and placed as a group in a plastic cage ($17.8 \times 28 \times 12.8$ cm) lined with bedding material and placed atop a heating pad maintained at a temperature of 33 ± 0.5 °C (Days 1–5) or 30 ± 0.5 °C (Days 6–14) everyday and returned to their home cages after 15 min (Litvin et al., 2010). Behavioral experiments began when voles were 90 days old.

Behavioral Testing 1: Open-Field Test

Spontaneous motor activity and anxiety-like behavior were assessed in an open field ($50 \times 50 \times 50$ cm) illuminated by six 60-W lamps mounted 2 m above the apparatus (Carneiro et al., 2005; Cullen, Burne, Lavidis, & Moritz, 2013; Ernsberger, Azar, & Iwai, 1983). The open field was divided into 25 squares (nine central and 16 peripheral). Adult male offspring were placed individually into the center of the open field for 5 min. The time spent in the central and peripheral zones, total distance covered during the experiment, and the number of crossings between squares were automatically recorded by a digital video system, and anxiety-related indicators analyzed by PC-compatible software (VideoMot 2, TSE Systems, Bad Homburg, Germany) included the frequency and time of entry into the central area, total distance, and number of crossings. The apparatus was cleaned with 70% ethanol after each test, and the interval between experiments was 30 min.

Behavioral Testing 2: Elevated Plus-Maze Test

After all open-field tests were completed, elevated plus-maze tests were started the next day. The open arm and closed arm of the elevated plus-maze test apparatus were placed 55 cm above the floor with movable pulleys at the bottom. The mandarin voles were moved out from the cage and placed at the connection of the open and closed arms, and their activities in the elevated plus-maze test were recorded by an overhead video camera for 5 min and coded later by a blind observer. The time and frequency of activities in the open and closed arms and the total distance were assessed as indicators of anxiety-related behavior (Lister, 1990; Rodgers & Dalvi, 1997). Voles were returned to their cage when the experiment finished and the apparatus was cleaned with 70% ethanol after each animal.

Measurement of E_2 and T Serum Levels

To avoid the effect of acute-stress-hormone measurement on data, blood samples were collected 2 days after behavior tests, from the anesthetized adult male retro-orbital sinus between 8:00–10:00 a.m. All serum samples were centrifuged from blood at 3,000 rpm for 10 min at room temperature. Serum samples were stored at −80 °C until assays were conducted. Serum T and E_2 concentration assays were measured by using enzyme-linked immunosorbent assay (ELISA; Nieminen et al., 2003). Serum samples were diluted 1:10 to measure T and E_2 levels (He & Tai, 2009). First, the prepared sample and the standard were added to dishes individually and then incubated for 30 min at 37 °C. Second, the dish was washed with solution four times, and a horseradish peroxidase (HRP)-blending agent was added in and incubated for 30 min at 37 °C. Last, after the dish was washed four times, color developing agents A and B were added. After 15 min incubation at 37 °C, the reaction was stopped using a stop solution. The optical density (OD) was determined at 450 nm using a microplate reader (Bio-Tek, Winooski, VT) and taking the blank well as zero. Variation between duplicate values was less than 5%.

Immunohistochemistry

The brains were collected at the same time as the blood; ERα and AR expression were also examined 2 days after behavioral testing (He, Yu, & Wu, 2013). Voles were deeply anesthetized and perfused with 0.1 molar (M) of phosphate-buffered solution (PBS, pH 7.4) and 4% paraformaldehyde in 0.1 M PBS. The brain was removed within 3 min and placed in 4% paraformaldehyde overnight. Prior to dissection, brains were immersed in 30% sucrose until saturated. Coronal sections (40 μm) were cut on a cryostat, and consecutive sections were collected in two vials containing 0.01 M PBS for two different immunohistochemical stainings. The ERα (sc-542; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and AR antibodies (Ba0004, Neomarkers, Inc., Fremont, CA) are affinity-purified rabbit polyclonal antibodies raised against a peptide and mapped at the C-terminus of mouse origin.

Floating sections were processed using primary antibody and streptavidin and peroxidase methods (Bao & Su, 1991). We incubated the sections for 7 min with 3% H_2O_2 and then washed each of them twice for 10 min with distilled water. We shrank the tissue in 0.01 M PBS. Sections were preincubated for 90 min with normal goat serum (SP-0023, Shanghai Ding Jie Biotechnology, Inc., Shanghai) and incubated at 4 °C overnight with the primary antibody solution (both ERα and AR antibodies, 1:100) diluted by an antibody diluent (0.01 M PBS containing 20% bovine serum albumin and 1.7% Trition X-100: a surfactants). The next day, sections were washed four times for 5 min each with 0.01 M PBS and incubated for 60 min in a 37 °C water bath with biotinylated goat antirabbit antibody (SP-0023), followed by another round of four washes for 5 min each with 0.01 M PBS. After 60 min of incubation with streptavidin/horseradish peroxidase (S-A/HRP) and four washes for 10 min each with 0.01 M PBS, sections were stained with 3,30-diaminobenzidine tetrahydrochloride (DAB) to visualize immunoreactivity. Because ERα and AR were included in nuclei, we counted stained nuclei using an Olympus microscope (Tokyo, Japan).

Slides were randomized and coded for microscopic analysis so that counters were blinded to experimental treatment. The number of cells that showed immunoreactivity was quantified by eye per standard area (200×200 lm) using grid sampling. We counted the number of ERα-IRs and AR-IRs in the BNST, mPOA, MeA, PVN, and supraoptic nerve (SON) in 40,000 μ^2. We selected these areas of the brain because they are involved in anxiety (He, Wu, & Yu, 2014; He, Zhang, Shi, & Wang, 2008; Song et al., 2010). Different brain areas were determined according to Nissl-stained brain sections from mandarin voles and a stereotaxic atlas of the rat brain (Bao & Su, 1991).

For each brain nucleus, three representative sections from anterior to posterior and anatomically matched between subjects were chosen and counted to minimize variability. Individual mean values for each animal were obtained by counting positive neurons bilaterally in three sections from each nucleus. Counts were performed separately for each hemisphere, and results were averaged between hemispheres. The left hemisphere was determined from the right hemisphere according to morphological characteristics of the brain surface: After the brain was removed within 3 min, we cut off a small part of the cortex in the left hemisphere and the right hemisphere as a template to discern the left hemisphere from the right hemisphere. Sections were chosen by correspondence to the reference atlas plate instead of the level or intensity of ERα-IR or AR-IR labeling. All immunohistochemistry procedures included negative controls (the primary antibody was not added). A trained experimental rater blinded to experimental treatment counted positive neurons for all subjects. Chosen sections were photographed with a Nikon camera (Tokyo, Japan) attached to a Nikon microscope.

Statistical Analysis

All data were analyzed using one-sample Kolmogorov–Smirnov tests. These data, including behaviors (time spent, distance traveled, number of crossings, and number of entries), ERα-IRs and AR-IRs in the five brain regions (BNST, MPOA, MeA, PVN and SON), and E_2 and T serum levels were normally distributed, and a two-way factorial analysis of variance (ANOVA) was required to analyze the relationship between mother (PS) and offspring (postnatal short-term maternal separation). If a significant difference was found for all data, post hoc multiple comparisons were conducted using the Tukey method. The degree of relationship between serum E_2 and T levels and locomotor activity, the distribution of ERα, the time in the center area of the open field, the distribution of AR, and time spent in the open-arm elevated plus-maze test were assessed by calculating correlation coefficients using a two-tailed Pearson product–moment statistic. All data are presented as mean ± *SE* and significance was set at $p < .05$. Statistical analyses were conducted using SPSS 10.0 (IBM/SPSS, Chicago, IL).

Results

Behavioral Testing 1: Open Field

The two-way ANOVA revealed a significant effect of prenatal handling for time spent in the central area, number of crossings, and total distance. However, postnatal handling did not changed significantly for time spent in the central area, number of crossings and total distance. The interaction between both factors was significant for time spent in the central area, number of crossings and total distance. According to post hoc tests, the PS/NH spent less time in the center area, number of crossings and total distance of the open field compared with all other groups ($p < .05$). There were no significant differences between the PC/NH, PC/H, and PS/H groups in spending time in the central area, number of crossings, or total distance in the open open-field test ($p > .05$; Table 1; Figure 1 and 2).

Behavioral Testing 2: Elevated Plus-Maze Test

Prenatal handling had significant effect on total distance, time spent in the open arm, number of entries to the open arm, and

Table 1
Main and Interaction F-Statistic Values for the Analyses

	Two-way ANOVA					
	Prenatal handling		Postnatal handling		Interaction between both factors	
Variable	*F*	*P*	*F*	*P*	*F*	*P*
Time spent in the central area in the OF	4.378	.037	2.583	.081	4.579	.033
Number of crossings in the OF	4.320	.038	1.889	.151	4.420	.036
Total distance in the OF	4.105	.040	2.132	.095	4.632	.032
Total distance in the EPM	4.735	.022	2.735	.073	4.645	.023
Time spent in the open arms in the EPM	4.258	.038	1.978	.136	4.878	.015
Number of entries to the open arm in the EPM	5.351	.008	1.784	.152	5.251	.008
Number of entries to the closed arm in the EPM	5.155	.009	2.254	.091	4.834	.021
E2 in serum	4.351	.037	2.854	.069	4.843	.020
T in serum	5.758	.006	2.925	.065	5.553	.007
ERα-IRs in the MeA	7.767	.002	2.767	.070	8.643	<.001
ERα-IRs in the mPOA	9.257	<.001	3.027	.056	9.077	<.001
ERα-IRs in the BNST	8.584	<.001	2.784	.070	8.594	<.001
ERα-IRs in the PVN	7.952	<.001	2.087	.121	7.967	<.001
ERα-IRs in the SON	8.027	<.001	1.781	.162	9.151	<.001
AR-IRs in the MeA	6.697	.003	2.871	.068	4.643	.023
AR-IRs in the mPOA	8.047	<.001	2.924	.065	17.077	<.001
AR-IRs in the BNST	8.013	<.001	2.902	.066	25.594	<.001

Note. E_2 = estradiol; T = testosterone; ERα = estrogen receptor-α; AR = androgen receptor; IRs = immunoreactive neurons; OF = open field test; EPM = elevated plus-maze test; PVN = hypothalamic paraventricular nucleus; SON = supraoptic nucleus; BNST = bed nucleus of stria terminalis; mPOA = medial preoptic area; MeA = medial amygdaloid nucleus.

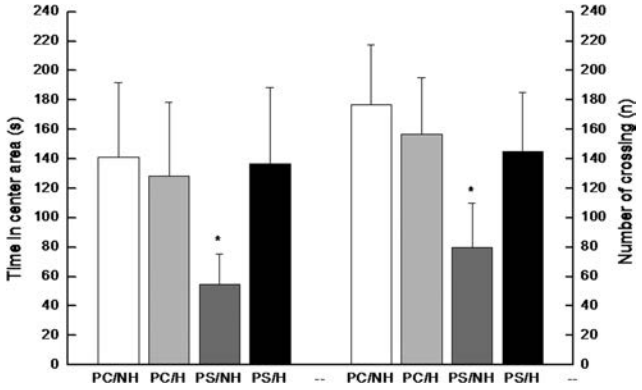

Figure 1. Time spent in the central area and number of crossings in the open-field test. PC/NH: control adult male offspring without postnatal handling; PC/H: control adult male offspring with postnatal handling; PS/NH: prenatal handling without postnatal handling; PS/H: prenatal handling with postnatal handling. The PS/NH spent less time in the center area and made fewer crossings over the open field than all other groups ($p < .05$). There were no significant differences between the PC/NH, PC/H, and PS/H groups in time spent in the central area ($p > .05$) and number of crossings ($p > .05$). All data represent the mean ($\pm$ *SE*).

number of entries to the closed arm. Postnatal handling did not significantly change total distance, time spent in the open arm, number of entries to the open arm and number of entries to the closed arm. The interaction between both factors was significant for total distance, time spent in the open arm, number of entries to the open arm, and number of entries to the closed arm. According to post hoc tests, the PS/NH group moved less total distance, spent less time in the open arm, decreased the number of entries to the open arm, but increased number of entries to the closed arm of the elevated plus-maze compared with all other groups ($p < .05$). There were no significant differences between the PC/NH, PC/H, and PS/H groups in moving total distance, time spent in open arms, and number of entries in the open arm and closed arm ($p > .05$; Table 1; Figures 2, 3, and 4).

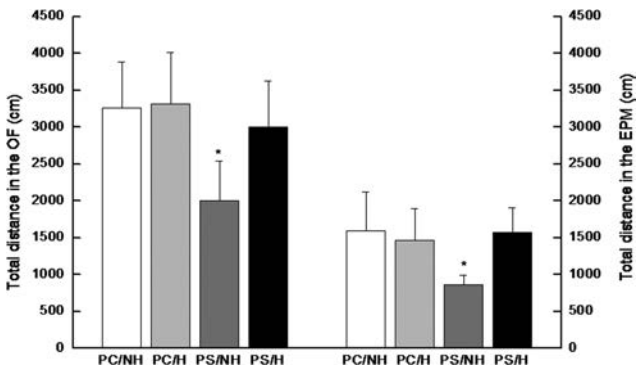

Figure 2. Total distance in the open field and elevated plus-maze test. The PS/NH moved less total distance of the open field and elevated plus-maze compared with all other groups ($p < .05$). There were no significant differences between the PC/NH, PC/H and PS/H groups in moving total distance of the open field and elevated plus-maze ($p > .05$).

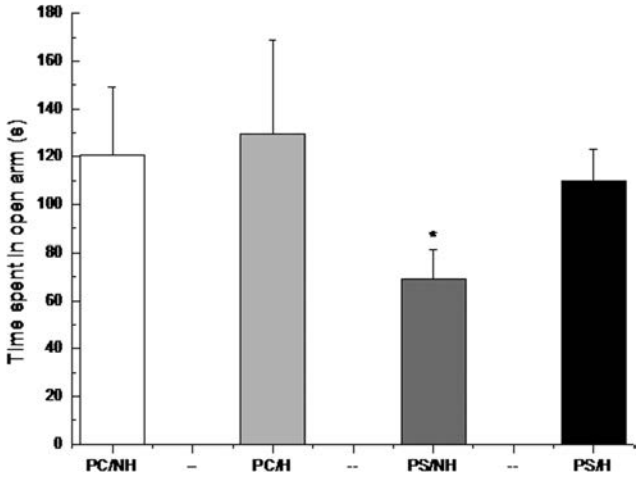

Figure 3. Time spent in the open arm. The PS/NH group spent less time in the open arm of the elevated plus-maze than all other groups. There were no significant differences between the PC/NH, PC/H, and PS/H groups in time spent in the open arm. All data represent the mean ($\pm$ *SE*); * $p < .05$.

E2 and T in Serum

Two-way ANOVA revealed a significant effect of prenatal handling for E_2 and T levels. However, postnatal handling did not change significantly for E_2 levels or T levels. The interaction between both factors was significant for both E_2 and T levels. According to post hoc tests, the serum E_2 level of the PS/NH group was lower than all other groups ($p < .05$), and the serum T level of the PS/NH group was also lower than all other groups ($p < .05$). There were no significant differences between the PC/NH, PC/H, and PS/H groups in E_2 and T serum levels ($p > .05$; Table 1; Figures 5 and 6).

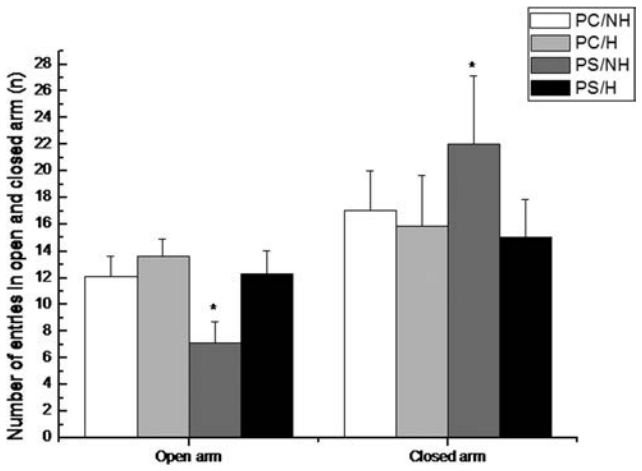

Figure 4. Number of entries into the open and closed arms. The PS/NH group's number of entries into the open arm decreased, but its number of entries into the closed arm of the elevated plus-maze increased compared with all other groups. There were no significant differences between the PC/NH, PC/H, and PS/H groups in the number of entries to the open arm and closed arm. All data represent the mean ($\pm$ *SE*); * $p < .05$.

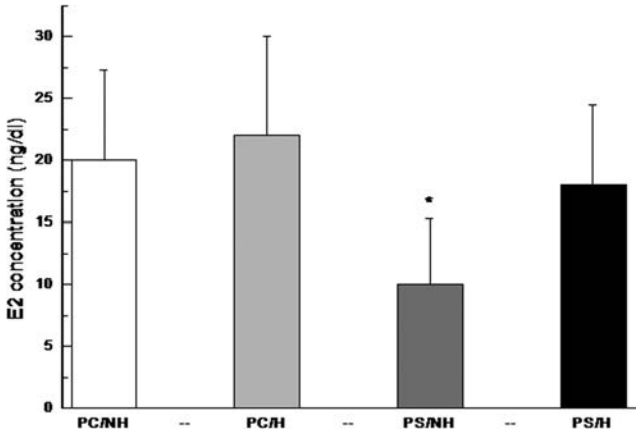

Figure 5. Effects of PS and short-term maternal separation on plasma E_2 levels in adult male offspring. Serum E_2 levels of the PS/NH group were lower than all other groups, and the serum T level of the PS/NH group was also lower compared with all other groups. There were no significant differences between the PC/NH, PC/H, and PS/H groups in E_2 serum levels. All data represent the mean (± *SE*); * $p < .05$.

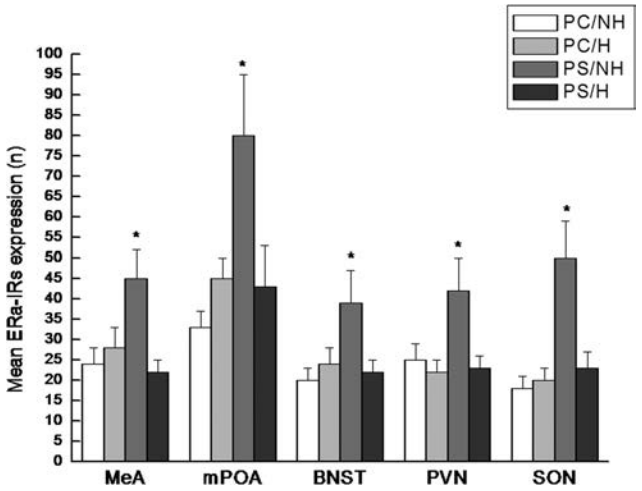

Figure 7. Mean (± *SE*) expression of ERα-IRs after PS and short-term maternal separation. The number of ERα-IRs in the MeA, mPOA, BNST, PVN, and SON in the PS/NH group was significantly increased compared with all other groups. There were no significant differences between the PC/NH, PC/H, and PS/H groups in the number of ERα-IRs in the MeA, mPOA, BNST, PVN, and SON. All data represent the mean (± *SE*); * $p < .05$.

Immunohistochemistry

Prenatal handling had an effect on the number of ERα-IRs in the MeA, mPOA, BNST, PVN, and SON; postnatal handling did not have such an effect. The interaction between both factors was significant for the number of ERα-IRs in the MeA, mPOA, BNST, PVN, and SON. The post hoc test showed that the number of ERα-IRs in the MeA, mPOA, BNST, PVN and SON in the PS/NH group was significantly increased compared with all other groups ($p < .05$). However, there were no significant differences in the number of ERα-IRs in the MeA, mPOA, BNST, PVN, and SON in the PC/NH compared with the PC/H and PS/H groups in the five brain regions ($p > .05$; Table 1; Figures 7 and 8).

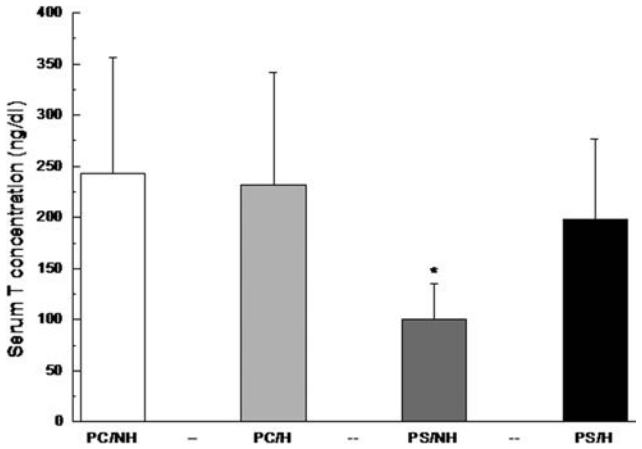

Figure 6. Effects of PS and short-term maternal separation on plasma testosterone levels in adult male offspring. Serum T level of the PS/NH group was lower than all other groups. There were no significant differences between the PC/NH, PC/H, and PS/H groups in T serum levels. All data represent the mean (± *SE*); * $p < .05$.

We did not find AR-IRs in the PVN and SON. Two-way ANOVA showed that the prenatal handling affected the number of AR-IRs in the MeA, mPOA, and BNST, whereas postnatal handling did not have the same affect. The interaction between both factors was significant for the number of AR-IRs in the MeA, mPOA, and BNST. The post hoc test showed that the number of AR-IRs in the MeA, mPOA, and BNST in the PS/NH group was significantly reduced compared with all other groups ($p > .05$). However, there were no significant differences in the number of AR-IRs in the MeA, mPOA, or BNST in the PC/NH compared with the PC/H and PS/H groups in the three brain regions ($p > .05$; Table 1; Figures 9 and 10).

Discussion

Prenatal Restraint Stress Increases Anxious Behavior in Adult Male Offspring

In this study, PS males spent less time in the center of the open field and traveled less (Figures 1 and 2). Decreased locomotor activity in these paradigms often reflects increased anxiety rather than some primary effect on locomotion (McKinney et al., 2008). To further assess anxious behavior in PS animals, an elevated plus-maze test, which is a well-validated and conventional test for anxiety-related behavior in animals, was used (Lister, 1987). The result showed that PS animals spent less time engaging in the open arms of the elevated plus-maze test, they made fewer entries into the open arms, and their total distance traveled was lower in the elevated plus-maze test (Figures 2–4). There are data to show that an increase in anxiety is associated with a significant decrease in exploratory behavior in the open arms of the maze, and that the number of entries into the closed arms or the total number of arm entries is a measure of locomotor activity (Shum, Ko, Lee, Kaang,

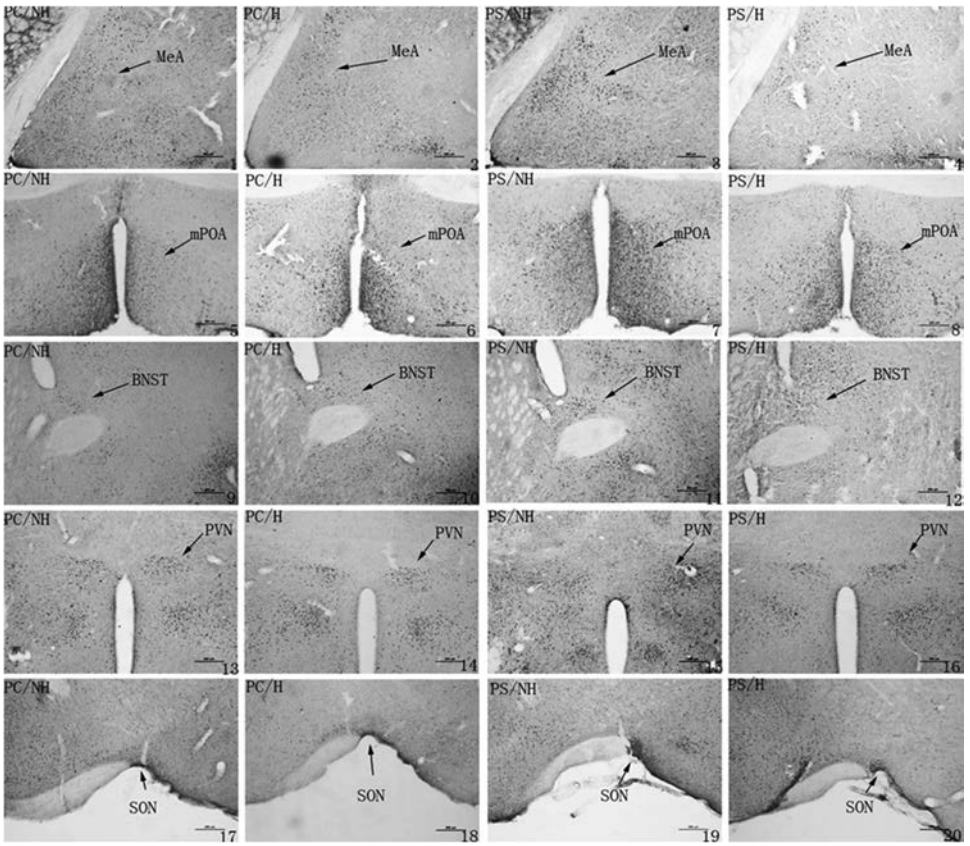

Figure 8. Position of ERα-IRs in the MeA, mPOA, BNST, PVN, and SON of male voles in the PC/NH, PC/H, PS/NH, and PS/H groups.

& Zhuo, 2005). Thus, all behavior results were indicative of increased anxiety-like behavior: it indicates that, during this stress experience, fetal brain growth is affected and has long-term effects on behaviors. These results are consistent with work on polygynous rat offspring and monogamous guinea pigs (Marrocco et al., 2012; Kapoor & Matthews, 2011) and reveal that prenatal restraint stress is associated with an increased risk of anxiety in adults (McEwen, 2012), regardless of monogamous or polygamous social systems. Previous studies in rats have shown that early PS can cause long-lasting changes in neuroplasticity that result in an increased risk in psychological disorders and stress-related diseases in adult life (Lupien, McEwen, Gunnar, & Heim, 2009). The offspring of pregnant voles receiving daily restraint stress during the last 2 weeks of pregnancy showed decreased serum T levels, and this is consistent with a reduction of serum T levels in rats and guinea pigs in response to high-intensity chronic stimuli (Kapoor & Matthews, 2011).

In normal gestation, male rat fetuses show a surge in T levels on the 18th and 19th gestational days, but such a surge is absent in male fetuses when mothers undergo stress during pregnancy (Ward & Weisz, 1980). Chronic PS increases the level of corticosterone and reduces gonadotropin in serum, inhibiting the activity of the hypothalamic–pituitary–gonadal (HPG) axis and reducing testicular weight (Chen Cárdenas et al., 2013). Stress during gestation can induce long-term effects on the reproductive systems of male offspring (Arena & Pereira, 2002; Pallarés et al., 2013), thereby affecting their T levels.

We found a negative association between prenatal restraint stress and E_2 levels in serum (see Figure 5), which is inconsistent with our previous work showing a positive association between prenatal treatment and estrogen levels in serum (He, Tai, et al., 2012). This may be because the previous experiment used female offspring and involved ethanol exposure as the stressor. Ethanol is a central nervous system depressant and can cross the placenta and directly affect fetal brain development via several mechanisms. Further, prenatal ethanol stress can increase the aromatization of T, so ethanol increases estrogen levels in serum (He, Tai, et al., 2012; Hilakivi-Clarke, Raygada, & Cho, 1997). However, why prenatal restraint stress decreases serum estrogen levels in adult male offspring is not yet completely understood. It may be that chronic PS inhibits the HPG axis, which reduces plasmatic luteinizing hormone and testicular weight (Chen Cárdenas et al., 2013), thus reducing the secretion of E_2 from testicular cells. PS can also decrease the activity of the steroid aromatase in the male fetal brain (Weinstock, 2005). However, the numbers of ERα-IRs in the BNST, mPOA, MeA, PVN, and SON were enhanced in males exposed to prenatal restraint stress in our study (Figure 7 and 8), and the BNST, MeA, PVN, and SON have been demonstrated to be involved in anxiety (Lee, Macbeth, Pagani, & Young, 2009). ERα is widely expressed throughout most E_2-sensitive tissues.

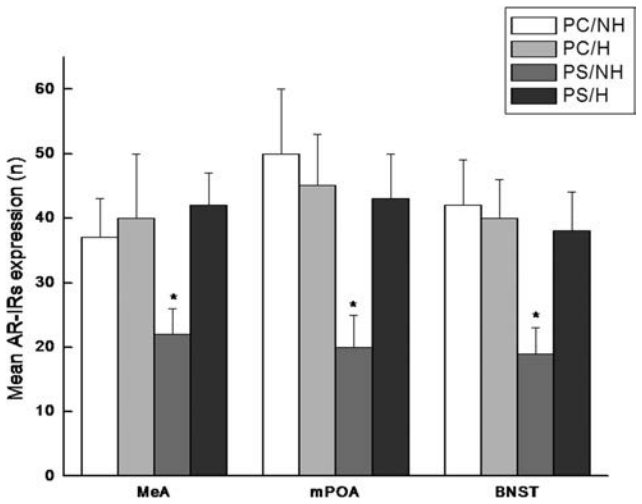

Figure 9. Mean (± *SE*) expression of AR-IRs after PS and short-term maternal separation. The number of AR-IRs in the MeA, mPOA, and BNST in the PS/NH group was significantly reduced compared with all other groups. There were no significant differences between the PC/NH, PC/H, and PS/H groups in the number of AR-IRs in the MeA, mPOA, and BNST. All data represent the mean (± *SE*); * $p < .05$.

There is differential expression of ERα in some regions, as well as differential brain distribution of changes in ERα that may indicate differential responsiveness to estrogen (Jensen, Jacobson, Walf, & Frye, 2010). Brain regions, including the mPOA, are associated with animal anxiety (Song et al., 2010; He, 2014), and ERα levels are related to anxiety-like behavior (Tsai, Wang, Hong, & Chiu, 2003). ERα activation increases fear and anxiety behavior (Morgan & Pfaff, 2001). In the present experiment, E_2 levels in serum of adult male offspring that underwent PS were lower than males in the PC/NH, PC/H, and PS/H groups, but the number of ERα-IRs in the BNST, mPOA, MeA, PVN, and SON increased (Figures 4, 5, and 6). E_2 levels in serum were lower, and amounts of ERα in brain regions were higher, consistent with other studies (Oliveira et al., 2004). ERα is thought to modulate the activity of corticotropin-releasing factor (CRF). Increased numbers of ERα-IRs in these brain regions raise the possibility that disturbed balance of CRF may affect the activity of the HPA axis and result in anxiety-like behavior (He, Tai, et al., 2012).

We found that serum T levels significantly declined in adult male offspring whose mothers experienced prenatal restraint stress (Figure 6, 9 and 10) and AR-IRs in the MeA, BNST, and mPOA were also fewer in adult male offspring whose mothers experienced prenatal restraint stress. This is consistent with our previous study that decreased T levels in serum and ARs in the central nervous system can result in the development of anxiety-like behavior (He, 2014). The amygdaloid region is a relatively complicated part of the brain and is related to anxiety-like behavior (LeDoux, 2000). It receives information input from the cerebral cortex and subcortical regions and integrates signals from other brain regions including, the BNST, mPOA, PVN, and SON, which ultimately results in expressed anxiousness in animals (Lee et al., 2009). Key brain regions including the BNST, mPOA, PVN, and SON have a high density of AR expression (Williamson & Viau, 2007), and changes in AR-IRs in these areas would likely lead to changes in anxiety. Hence, the significant decline in T levels in serum and the number of AR-IRs in the BNST, mPOA, and MeA increases the risk of anxiety-like behavior in adult male offspring. We speculate that this pattern, i.e., the decrease in T, is likely to cause down regulation of AR in these brain regions and is consis-

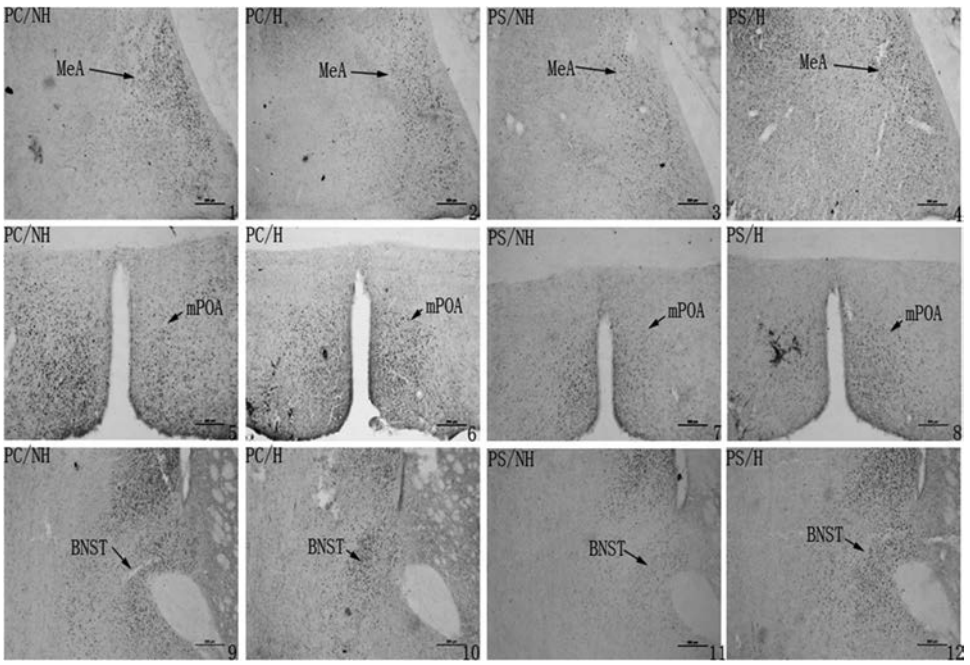

Figure 10. Position of AR-IRs in the MeA, mPOA, and BNST of male voles in the PC/NH, PC/H, PS/NH, and PS/H groups.

tent with previous findings that declining T levels in males can decrease or completely eliminate AR-IRs in these brain regions (Arteaga-Silva, Rodríguez-Dorantes, Baig, & Morales-Montor, 2007; He, Zhang, & Guo, 2012). Through ARs, T plays an important role in certain brain regions regulating anxiety-related behavior. For example, anxiety related to T circulation is dependent on ARs (Zuloaga et al., 2008; Zuloaga, Jordan, & Breedlove, 2011) and supports the notion that ARs may play a role in the regulation of anxiety in rodents. However, few AR-IRs were identified in the PVN and SON in our experimental groups. Different distributions of ARs are found across different brain regions and species (Bingham et al., 2006). So far, there is only one report suggesting that prenatal restraint stress may affect AR levels in the mPOA (Ward, Bennett, Ward, Hendricks, & French, 1999), thus, the relationship between the distribution of ARs in other brain regions and prenatal restraint stress is unclear. Prenatal restraint stress can induce long-term effects on the reproductive system of male offspring (Arena & Pereira, 2002; Pallarés et al., 2013). Therefore, prenatal restraint stress may alter AR expression or the HPA axis and affect ARs and T.

Postnatal Short-Term Maternal Separation Reverses PS-Induced Anxious Behavior in Adult Male Offspring

Our open-field test and elevated plus-maze test results show there was no effect of anxiety in the postnatal handling-alone group (Figures 1–4), which is consistent with Lehmann and colleagues' findings that postnatal maternal separation does not affect rat-offspring anxiety (Lehmann et al., 2000). Short periods of separation or handling regimens appear to be beneficial to pups (Lehmann et al., 2000). Although prenatal restraint stress led to reduced motor activity and increased anxiety-like behavior in adult male offspring, and postnatal short-term maternal separation reversed PS-induced anxiety, the result is consistent with findings that increased motor activity and decreased anxiety-like behavior in adult male offspring follow short-term maternal separation (Vallée et al., 1997). In the present test, T levels in serum in the PS/H group were consistent with recent studies demonstrating that postnatal short-term maternal separation induces changes in serum T levels in male rats (Chen Cárdenas et al., 2013). During the critical period of fetal gonadal development, the effect of PS would occur through the reducing activity of aromatase in the hypothalamus (Weinstock, 2005) and 5 alpha-reductase activity in the cerebral cortex and hypothalamus of male fetuses one day after the last session of stress (Ordyan & Pivina, 2005) and D-3-β-hydroxysteroid dehydrogenase in the testis (Orth, Weisz, Ward, & Ward, 1983). E2 and T levels in serum in the PS/H group had no significant differences with the PC/NH in the present test that may be occurring by this mechanism which these animals are postnatally stimulated to cause luteinizing hormone and the size of the testis increase and T levels return to normal (Chen Cárdenas et al., 2013). T can be exerted either directly through the AR or indirectly through aromatization to E2 (He, Zhang, et al., 2012). Therefore, E2 and T levels in serum in the PS/H group had no significant differences with the PC/NH. The PS/H voles spent more time, a greater number of crossings, and moved further (i.e., the whole distance) through the open field than the PS/NH group during the test, and moved further than the PS/NH group in the elevated plus-maze test (Figures 1–4). These results are consistent with the findings that have suggested a positive relationship between plasma testosterone levels and locomotor activity in laboratory-bred male meadow voles (Perrot-Sinal, Innes, Kavaliers, & Ossenkopp, 1998). The presently described relationship between activity and testosterone has been inferred in laboratory-reared animals, mainly on the basis of castration and hormone-replacement manipulations. For instance, injections of testosterone induced significant increases in activity levels in castrated male voles compared with vehicle-injected castrates (Rowsemitt, 1989).

It is known that postnatal short-term maternal separation can cause further changes in an HPA axis already altered by PS, and these changes are likely to influence anxious reactivity (Weinstock, 1997). However, studies on the effects of maternal separation on anxiety have yielded variable results (Kalinichev, Easterling, Plotsky, & Holtzman, 2002) and seem to be related to maternal separation procedures, testing conditions, and stress varieties. For example, adult wild-type offspring fostered by mice with variational biological clocks have been found to exhibit increased anxiety-related behavior (Koizumi, Kurabayashi, Watanabe, & Sanada, 2013). However, in Wistar rats, prolonged maternal separation can lower anxious responses, decrease anxiety, and reverse the effects of adverse conditions on their behavior (Roman, Gustafsson, Berg, & Nylander, 2006). Likewise, pups exposed to chronic unpredictable maternal separation combined with maternal stress develop some resilience to stress when they grow up (Franklin & Mansuy, 2011).

AR has a role in anxiety-like behavior, but the mechanism by which AR activation reduces anxiety is yet to be established. One possibility is that AR stimulation may activate GABAergic drive, known to reduce anxiety (Reynolds, 2008). Here, the distribution of ERα-IRs was also reversed to normal levels in the BNST, mPOA, MeA, SON, and PVN by postnatal short-term maternal separation, which may be related to decreased anxiety-like behavior in adult male offspring.

Postnatal maternal separation causes changes in the mother's behavior to a certain degree, such as increased care of offspring (Levin & Stern, 1975) and increased licking behavior (Barbazanges et al., 1996). This environment-dependent variation in maternal behavior also alters behavioral and HPA responses to stressors in adult offspring (Macrì & Würbel, 2006). Mother's behavior may be a result of a number of neuropeptides (e.g., oxytocin and arginine vasopressin), gonadal steroids (e.g., testosterone), and other neurotransmitters being released at parturition and during suckling, promoting the development and maintenance of maternal bonding and maternal care, and further influencing the expression of locomotor activity and exploratory behavioral responses to stress (Curley, Jensen, Mashoodh, & Champagne, 2011).

Conclusion

Despite the limitations in this study, we were able to report the relationship between perinatal stress effects on later anxiety and hormone secretion and ERα and AR in male mandarin voles. In addition, there were some consideration of the possible roles of and changes in ERβ. As such, we do have another article forthcoming that considers these roles and changes. For the current study, we examined the combined effects of PS and early postnatal maternal separation, which provided insights into the complex relationship between the two. Our data indicated an increase in anxiety-like behaviors in open-field and elevated plus-maze tests

in PS male adults, which was associated with significantly lower estradiol and testosterone levels in serum; an increased number of ERα-IRs in the BNST, mPOA, MeA, PVN, and SON; and a reduced number of AR-IRs in the MeA, mPOA, and BNST. Only early postnatal maternal separation effects were similar to the control group; they did not produce anxiety-like behavior. However, early postnatal maternal separation reversed prenatally induced changes in estradiol and testosterone levels in serum and altered ERα-IRs and AR-IRs in the brain. These data suggest that anxious changes in adults may be governed by early environmental factors and their interaction, because changes in estradiol and testosterone levels in serum and the distribution of ERα-IRs and AR-IRs in the brain result in behavioral changes related to anxiety well into adulthood.

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