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Research report

Early weaning augments neuroendocrine stress responses in mice

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Abstract

Weaning is one of the most important events in early mammalian life. We investigated the influence of early weaning on the development of neuroendocrine responses to stress in mice. Our study indicated that precocious weaning augments anxiety and aggressiveness in mice, and the lack of mother–pup interaction from postnatal days 15–21 may account for this phenomenon. A litter of Balb/cA mice was divided into two groups, with one group weaned at postnatal day 14 (early weaned) and the other at day 21 (normally weaned). Baseline levels of corticosterone, corticosterone response to the moderate stress of exposure to the elevated plus maze test, and hippocampal glucocorticoid receptor (GR) mRNA expression levels in the mice were assayed at the age of 3, 5, and 8 weeks. At 8 weeks, basal corticosterone levels in early-weaned males were higher than those of normally weaned males. Also at 8 weeks, the early-weaned mice showed a higher anxiety level in the elevated plus maze test, and responded with greater levels of corticosterone secretion than the normally weaned mice. GR expression in the early-weaned mice was higher at 3 weeks but lower at 8 weeks. These GR changes were observed only in male mice. These results suggest that early weaning increases anxiety and enhances neuroendocrine responses to stress and thus modulates the development of the neuroendocrine stress system.

Keywords: Early weaning; Corticosterone; Anxiety; Glucocorticoid receptors; Development

1. Introduction

Events in the early stages of life are known to influence the behavior of adult individuals. Maternal deprivation during the first 10 days of the neonatal period, when the mother rat shows intensive nursing behaviors, such as grooming and licking, augments anxiety-related behaviors of the pups in adulthood, without influencing somatic growth [6,35]. In addition, maternal deprivation also lowers resistance to cancer [30] and enhances the neuroendocrine response to stressors [19,32,43].

For rats, a large body of evidence shows that maternal care received by the neonate has critical effects on the response of the neuroendocrine system to stress [5,7,37,48]. In monkeys, as in rats, infant monkeys raised by mothers foraging under unpredictable conditions displayed augmented secretion of corticotropin-releasing factor [9]. Individual differences in the neuroendocrine stress response are thought to be a risk factor for mental and cardiac stress disorders [3,18]. The maternal environment has been demonstrated to alter glucocorticoid receptor (GR) expression levels in the hippocampus, which is the key structure in the neuroendocrine stress feedback loop [2,37,41]. Offspring that were deprived of maternal care or received a lower level of maternal grooming showed a lower level of GR expression in the hippocampus, indicating a decrease in the negative feedback of glucocorticoids and a subsequent increase of glucocorticoid secretions in response to stress episodes. In addition to rat studies, it has been shown that manipulation of the maternal environment during the first 2 weeks of the neonatal period induces anxiety, as well as a stress endocrine response. For example, a single 24-h maternal deprivation [53] or daily 3-h deprivation [45] increased HPA activity in mice. Behaviorally, maternal deprivation induced greater anxiety in male mice than in females [51]. In addition, Holmes et al. demonstrated that the postweaning social environment influenced the anxiety levels in mice [25].

In abovementioned studies, maternal care was manipulated during the first 2 weeks of the neonatal period, i.e., the stress hypo-responsive period [34]. In the normal developmental process of rats, pups can eat, maintain body temperature, and evacuate by themselves from 13 days of age [47]. The neuroendocrine system becomes sensitive to stressors from that age throughout the weaning period [34]. Weaning is one of the

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most important events characterizing the early developmental stage. After weaning, the offspring must become nutritionally and behaviorally independent of their dam. Thus, the weaning procedure may well influence physiological and neurobehavioral development. Janus [27] reported that early-weaned rats showed a low rate of play-fighting behavior and low levels of exploratory behavior in adulthood. Early weaning has also been shown to influence social interactions of juvenile mice [55]. We recently found that precocious weaning augments anxiety and aggressiveness in mice and rats for a long period, which suggests that the parent–pup interaction during the late lactating period is important for behavioral development [28,29,42]. However, there is no information about the influences of early weaning on the stress neuroendocrine system.

In this study, we conducted experiments to determine the influence of early weaning on the neuroendocrine stress system. We examined changes of basal corticosterone levels and GR expression in the hippocampus during early developmental stages, as well as anxiety levels assessed by the elevated plus maze test.

2. Materials and methods

2.1. Animals

Balb/cA mice, originally obtained from Japan Clea Co. Ltd. (Yokohama, Japan) were used for the experiments. Male and female mice were pair-housed in cages (45 cm × 35 cm × 35 cm) for breeding. Food and water were provided *ad libitum*, and all animals were kept at constant temperature (23 ± 1 °C) and humidity (40 ± 5%) under a 12 h/12 h light–dark cycle (lights on at 0800 h). All experiments were conducted in accordance with guidelines from the "Policies Governing the Use of Live Vertebrate Animals" by the University of Tokyo, which is based on "The Public Health Service Policy on Humane Care and Use of Laboratory Animals" by Awardee Institution (revised May 1985) and "The National Institutes of Health Guide for the Care and Use of Laboratory Animals" (revised 1985) in the treatment of animals.

2.2. Weaning procedure

When a female mouse became pregnant, the male partner was removed, and the female was checked for birth every day. For each litter, the day of birth was designated postnatal day 0 (PD0). On PD2, the litters were culled to six or eight, with half the pups male and the other half female, to standardize the litter size. Throughout the nursing period, care was taken not to disturb the animals, except for a brief cage cleaning each week. On PD14, half the litter was separated from the dam and housed in cages (12.5 cm × 20 cm × 11 cm) in same-sex sibling groups of two or three. The early-weaned mice were fed ordinary adult pellets (MM-3; Funabashi No-jo, Co. Ltd., Chiba, Japan), as were the normally weaned mice after weaning. The normally weaned mice were weaned on PD21, the natural time of weaning, and housed in same-sex sibling groups of two or three in 12.5 cm × 20 cm × 11 cm cages. The cages were cleaned once a week, on a day at which all of the litters were not necessarily at the same postnatal stage. Both male and female mice were used in the following experiments, and they were housed in a group of two to three mice per cage with the same sex and same treated mice until the behavioral test and sampling. Two or three male and female mice from each litter were used for each group to minimize the inter-litter differences, and each mouse was tested once in the elevated plus maze test as well as blood sampling at either age.

2.3. Elevated plus maze test

The elevated plus maze test was conducted with pups at 3, 5, and 8 weeks during the light period under a dim red light using a standard plus maze apparatus (25 cm × 5 cm close arm with a wall 5 cm high) elevated 20 cm above the floor. The animal was placed in the neutral zone facing the opened arm, and its behavior was video-recorded for 15 min. The frequency of entry into the arms and duration in the arms were noted. In addition, the distance traveled in the maze was analyzed using an Ethovision computer tracking system (version 3.0; Noldus Co. Ltd., Wageningen, The Netherlands) and a computer-based behavior analyzing system (CIF12; Toyo Sangyo Co., Toyama, Japan). Two or three mice from each of 45 litters were used for each group in the elevated plus maze test to minimize the inter-litter differences, and each mouse was tested once at either age. The following numbers of animal were used: early-weaned males, 3 weeks ($n = 23$), 5 weeks ($n = 23$), and 8 weeks ($n = 19$); early-weaned females, 3 weeks ($n = 24$), 5 weeks ($n = 31$), and 8 weeks ($n = 22$); normally weaned males, 3 weeks ($n = 27$), 5 weeks ($n = 25$), and 8 weeks ($n = 18$); normally weaned females, 3 weeks ($n = 25$), 5 weeks ($n = 20$), and 8 weeks ($n = 18$).

2.4. Blood sampling

All the mice that had been assigned to blood sampling were subjected to the plus maze test beforehand (corticosterone response after stress) or afterward (basal level of corticosterone). Blood sampling for basal corticosterone levels was performed by cardiac puncture under ether anesthesia immediately after removing the mice from their home cage, where they were undisturbed, and every blood sampling was conducted within 1.5 min of removal in order to minimize the stress effects on the basal corticosterone level, as described elsewhere [4] (early-weaned males, 3 weeks ($n = 5$), 5 weeks ($n = 8$), 8 weeks ($n = 10$); early-weaned females, 3 weeks ($n = 6$), 5 weeks ($n = 8$), 8 weeks ($n = 21$); normally weaned male, 3 weeks ($n = 5$), 5 weeks ($n = 10$), 8 weeks ($n = 11$); normally weaned females, 3 weeks ($n = 5$), 5 weeks ($n = 12$), 8 weeks ($n = 19$)). To obtain the stress response of corticosterone, mice were subjected to the elevated plus maze test for 15 min, and their blood was collected 20 and 45 min after placing them in the maze (20 min: early-weaned males, 3 weeks ($n = 6$), 5 weeks ($n = 9$); 8 weeks ($n = 10$); early-weaned females, 3 weeks ($n = 10$), 5 weeks ($n = 8$), 8 weeks ($n = 17$); normally weaned males, 3 weeks ($n = 5$), 5 weeks ($n = 10$), 8 weeks ($n = 11$); normally weaned females, 3 weeks ($n = 13$), 5 weeks ($n = 8$), 8 weeks ($n = 15$); 45 min: early-weaned males, 3 weeks ($n = 15$), 5 weeks ($n = 16$), 8 weeks ($n = 17$); early-weaned females, 3 weeks ($n = 16$), 5 weeks ($n = 12$), 8 weeks ($n = 15$); normally weaned males, 3 weeks ($n = 13$), 5 weeks ($n = 13$), 8 weeks ($n = 12$); normally weaned females, 3 weeks ($n = 13$), 5 weeks ($n = 11$), 8 weeks ($n = 15$)). All blood collection was conducted between 1100 and 1700 h in an alternating manner. Plasma samples were frozen at −20 °C until assayed.

2.5. Corticosterone assay

Steroids were extracted from 10 μl of plasma and corticosterone levels were measured by specific enzyme immunoassays (EIA). Each well (ELIZA Plates 9018; Corning, New York, NY, USA) was coated with 100 μl of secondary antibody solution (anti-rabbit γ-globulin serum raised in goats, 5 μg/200 μl; Seikagaku Co., Tokyo, Japan) and incubated overnight at 4 °C. Non-bound antibodies were then removed from the wells by emptying and washing two times using a plate washer (IMMUNOWASH MODEL 1250; Bio-Rad, Hercules, CA, USA), and the plates were inverted to dry. Standard corticosterone (Wako Chemicals, Osaka, Japan) was diluted in the assay buffer (pH 7.2 sodium phosphate buffer containing bovine serum albumin at 1 g/l). Twenty-five-microliters aliquots of the standard or sample solutions, 100-μl aliquots of antiserum solution, and then 100-μl aliquots of labeled steroid hormones were sequentially pipetted into each well. The anti-corticosterone serum (the first antibody was raised in a rabbit, FKA420-E; COSMO Bio, Tokyo, Japan) was diluted 1,000,000-fold with assay buffer. Horseradish-peroxidase (HRP)-labeled corticosterone (FKA419) was obtained from COSMO Bio. The plates were covered and incubated overnight at 4 °C. Non-bound ligands were removed and a 150-μl aliquot of the substrate solution for HRP was added to each well and incubated for another 40 min at room temperature. The reaction was stopped by the addition of 50 μl 4N-H_2SO_4. The absorbance at 450 nm of sample and standard solutions was recorded with an automatic microplate reader (Bio-Rad model 550). The results of the assay were calculated by using the software Microplate Manager