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Maternal-infant separation impedes changes in feeding behavior during estrous cycle of rats

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Abstract: Traumatic and stressful events during childhood are associated with the development of eating disorders. We conducted an animal study to test if association stress in childhood affects ingestive behavior later in life by using female rats that have an adjusted estrous cycle. First, electrical impedance of the vagina was conducted to test estrous cycle adjustment. Second, the effects of 6 h per day maternal separation from birth to weaning, which models a psychologically stressful experience in childhood, was used to test feeding behavior during an ovarian cycle in female adult rats with matched estrous cycles. Food and water intake in maternal separated and non-separated rats was measured in each estrous phase. Non-separated rats showed periodical changes, but maternal separated rats showed no significant changes in food and water intake during an estrous cycle. An opposing tendency for food and water intake was seen between maternal separated and non-separated rats. These observations suggest that electrical impedance of the vagina showed the highest value in the estrous phase of rats housed in a reversed light-dark cycle, and maternal separation was found to disturb changes in feeding behavior during the estrous cycle.

Key words: eating disorders, electrical impedance of the vagina, estrous cycle, female rat, maternal separation

Introduction

Eating disorders are characterized by abnormal eating habits that may involve insufficient or excessive food intake to the detriment of an individual's physical and emotional health. It is unclear what causes eating disorders. Many patients with eating disorders, particularly those with bulimia nervosa have reported that they were abused in childhood [15, 40, 47, 48, 55]. Herzog [24] reported that many bulimia nervosa patients had broken families, as their parents either lived apart, or were divorced, or were bereaved. Kinzl *et al.* [31] reported that bulimia nervosa patients had an adverse family background. Gupta *et al.* [20, 21] reported that touch deprivation during the early childhood years played a role in

body image pathologies and was also seen in eating disorder patients. These results suggest that traumatic or stressful events in childhood may contribute to the development of eating disorders. However, the role of childhood trauma in the development of eating disorders is controversial [46]. A combination of biological, psychological, and environmental issues is implicated in the cause of eating disorders.

Studies on eating disorders and eating behaviors using animal models have been published previously [22, 23, 26, 27, 49, 54]. However, the majority of these studies used male animals, and a few core facts have been discovered from these experiments [4]. To clarify the underlying biology of eating disorders, one must also consider the use of female animals. However, research-

ers prefer not to use female animals because their estrous cycle can affect physiological, biological, and behavioral features, and may thus confound the data. A comprehensive survey of articles published in representative neuroscience journals revealed that the ratio of single-sex studies involving male versus female animals is almost 6 to 1 [4]. Animal studies for some specific fields of study would benefit from the use of female animals. Eating disorders have a large sex difference, as about 90% of human patients with eating disorders are female [36]. If female animals, such as female rats are to be used in experiments, then it is preferable that the female rats have synchronized estrous cycles. The estrous cycle is characterized by cyclical changes in the uterus, ovaries, and vaginal mucosa, and also results in changes in behavior and hormone levels [37, 44]. The rat estrous cycle consists of a 4- to 5-day cycle of estrous, metestrous, diestrous, and proestrous phases [32]. To determine the estrous cycle stage, vaginal smear and histological analyses have been used. However, invasive and stressful methods, such as taking a vaginal smear may induce stress or pseudopregnancy, thus a simple and quick method is needed to study stress-induced problems. Correlation of the electrical impedance of vaginal mucus (EIV) with the estrous cycle was reported in cattle [9], rats [1–3, 51], and other animals [18, 39]. It is possible to instantly measure impedance with a rat vaginal impedance instrument that can detect the optimum day for mating [33, 34]. We attempted to determine the associations of EIV and vaginal smear histological change.

Many investigators have attempted to develop animal models of childhood stress. Postnatal handling, early separation, and daily periodic maternal separation have been proposed as methods that produce psychological stress with little physical stress [29, 30, 35, 41, 43]. Normal mother-infant interactions are critical for growth and development in many mammalian species, and maternal separation is a profound stress that affects physiological and behavioral functions in the offspring [29, 30, 35, 41, 43]. We conducted a study to elucidate association stress in childhood with ingestive behavior in later life by using female rats with an adjusted estrous cycle. Food and water intake and estrous cycle were measured in maternal separated and non-separated female rats in later life.

Materials and Methods

Animals

For experiment 1, thirteen 8-week-old female Wistar rats were used (Keari Co., Osaka, Japan). All rats were housed in individual cages (22.0 × 21.0 × 30.0 cm) and maintained on a reversed 12-h light-dark cycle (lights on at 20:00 h). Rats were given CE-2 chow (24.8% crude protein, 4.4% crude fat, 3.5% crude fiber, 7.0% crude ash, 51.6% nitrogen-free extract, 8.7% water; 345.2 cal/100 g: Clea Co. Tokyo, Japan) and water using an auto water-supply system (Keari Co.)*ad libitum*. The animal room was maintained under constant temperature (22 ± 2°C) and humidity (55%). For a 1-week acclimation period, all rats were handled with the least amount of stress so as to not affect baseline measurements. Acclimation day 8 was defined as the experimental day.

For experiment 2, eight pregnant Wistar rats on gestation day 14 − 16 were used (Keari Co.). They were housed individually in polycarbonate maternity cages (34.5 × 40.3 × 17.7 cm), and were maintained on a reversed 12-h light-dark cycle (as above). Dams were given access to CE-2 chow and water *ad libitum*. The animal room was maintained under constant temperature and humidity (as mentioned above). Prior to birth, the dams were randomly divided into two groups: a maternal-separated (MS) group and a non-separated (NS) group. The day of birth was designated as postnatal day 0 (PD 0). Within 12 h of birth, the litters were culled to eight pups with equal numbers of male and female animals, when possible. From PD 1 − 21, pups in the MS group were immediately removed from their home cage at 09:00 h and were placed into another clean cage lined with a clean paper towel on a heating pad (35°C). After 6 h (at 15:00 h), pups were returned quickly to their home cages. During the same period, pups in the NS group were handled (rats were picked up and then immediately returned to their home cage) twice a day at 09:00 and 15:00 h at the same time that the MS rats were moved, to control for the handling of the rats. Except for the manipulation described above, all pups were left undisturbed for the first 3 weeks. Cage maintenance was not performed during this period. At PD 22, all pups were weaned and dams were removed from the home cage. Only female pups were used after this. All female pups in the MS group and NS group were divided into new cages. Pups of the same group were housed two to four per cage. Until the age of 8 weeks, pups were given

access to CE-2 chow and water *ad libitum* and were left undisturbed, except for regular cage maintenance. All rats reaching 8 weeks of age were housed in individual cages (22.0 × 21.0 × 30.0 cm). Animals were acclimated to the experimental room and experimental handling for 1 week. All rats were cared for in compliance with the Guidelines for Animal Experimentation of Osaka City University.

Measurement of electrical impedance of the vagina

We wanted to determine the estrous phase in an adjusted estrous cycle using a method that results in less stress to the female rats. EIV measurement was performed using Rat Vaginal Impedance Checker MK-11 (Muromachi Kikai, Tokyo, Japan). This is a diagnostic instrument that detects the mating phase [33, 34] by using impedance (similar to electric resistance) of the vaginal mucosa. All impedance measurements were performed at 14:00 h (in the dark phase). The impedance checker has a terminal with two electrodes. While holding the rat, the terminal was inserted into the vagina to the external os. Within 5 s the impedance was stabilized. The value of EIV was checked and recorded. After each measurement, the terminal was cleaned with alcohol and saline.

Vaginal smear

Vaginal smears were collected immediately after each EIV measurement. Vaginal smears were taken using a cotton swab. Contents of the swab were placed onto a glass slide, dried, and stained using a previously described method [38], and then examined under a microscope. The samples were classified into the four stages: proestrous, estrous, metestrous, and diestrous. Each type of epithelial cells and leukocyte was counted.

Experiment 1: association between EIV and vaginal smear

A possible correlation was examined between EIV and vaginal smear from each female rat. EIV and vaginal smear of 9-week-old female rats were examined during three separate consecutive periods of 4 days (at least 12 days). Based on a previous study [28], 9 weeks of age was determined. We previously observed that at 9 weeks of age maternal separation makes pups vulnerable, and likely to affect behavior. The estrous cycle phase was determined by vaginal smear, and the mean EIV in each phase was calculated. The highest EIV day was referred

to as day EH, and the estrous cycle phase was determined by vaginal smear.

Experiment 2: effect of maternal separation on food and water intake and estrous cycle

At 9 weeks of age, MS and NS rats were given free access to food and water. EIV of each rat was checked every day. The highest EIV day was defined as day EH, as mentioned in Experiment 1. Consecutive 3 EH days were defined as days E2, E3, and E4. During each 24-h time period, food and water consumption was measured between EH and E4.

Statistical analysis

All values are expressed as mean ± SEM. Correlation ratio and analysis of variance was used to measure correlation between EIV and estrous phase. Analysis of variance was used to compare the differences in food and water intake by the estrous cycle groups. The Tukey–Kramer test was used as post-hoc analysis to compare differences within each group. The Student's *t*-test was used for comparison of food and water intake of the MS group with those of the NS group. All statistical data were analyzed using the JMP 8 software package (SAS, Cary, NC, USA).

Results

Experiment 1

The results of the EIV experiment in each estrous phase determined by vaginal smear are shown in Fig. 1. η^2 (the square of correlation ratio: η) was 0.96, which showed high correlation between EIV and the estrous cycle. There were significant changes in EIV in each estrous period [F (3,48)=342.2, *P*<0.0001]. EIV in the first estrous phase (8.0 ± 0.3 kΩ) was significantly elevated compared with EIV in the other three phases (metestrous: 1.4 ± 0.1 kΩ, diestrous: 0.9 ± 0.0 kΩ, proestrous: 0.8 ± 0.1 kΩ; *P*<0.0001). EIV in the metestrous was significantly higher than EIV in diestrous and proestrous phases. The other two phases (diestrous and proestrous) were not significantly different from each other.

Cytological classification of vaginal smears on day EH is shown in Table 1. On day EH, 89.7% of rat vaginal smears presented as estrous phase.

Experiment 2
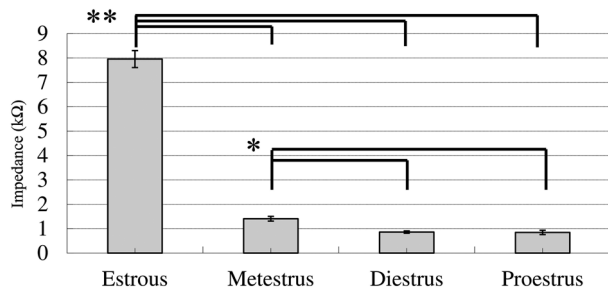
Body weight on day EH were 250.7 ± 10.3 g in MS

Fig. 1. Estrous cycle and vaginal impedance. Electric impedance of the vagina (EIV) for each estrous phase (estrous, metestrous, diestrous, proestrous). * Denotes statistical significance against other phase-specific EIV (*$P<0.05$, **$P<0.01$)

Table 1. Number of rats classified by vaginal smear in day EH

Classification	day EH
Estrous	26 (89.7%)
Metestrus	1 (3.4%)
Diestrus	0 (0.0%)
Proestrus	2 (6.9%)

Highest EIV (electrical impedance of the vagina) day was defined day EH in one estrus cycle.

group and 272.4 ± 9.6 g in NS group (mean $\pm$ SEM). There were no significant differences in the body weight between MS and NS group.

Daily food and water intake during one estrous cycle of MS and NS rats is shown in Fig. 2 (food intake) and Fig. 3 (water intake). NS rats exhibited decreased intake of food (F $(3,28)=5.81$) and water, (F $(3,28)=4.673$) on day E4 (mainly proestrous phase in this study), demonstrating a periodic change. However, MS rats showed no statistically significant changes in food and water intakes during one estrous cycle.

MS rats tended to take less food than NS rats, and the difference was statistically significant on day EH (mainly estrous phase; $P<0.05$). In contrast, MS rats drank more water than NS rats, and the difference reached statistical significance on day EH ($P<0.01$), E2 (mainly metestrus phase; $P<0.05$), and E3 (mainly diestrus phase; $P<0.05$). MS and NS rats had opposite tendencies regarding food and water intake.

All rats in MS group had 4-day estrous cycle by the elevation of EIV on day E5 again confirmed.
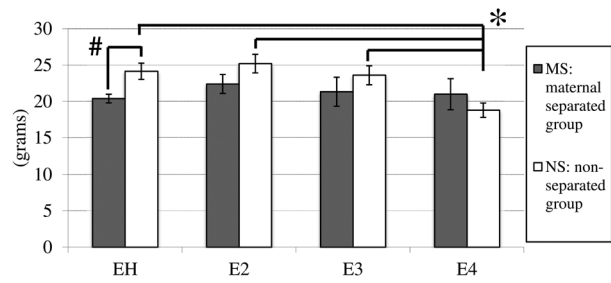

Fig. 2. Maternal separation and daily food consumption. Food consumption in each estrous phase of maternal separated (MS, gray column) and non-separated (NS, white column) female rats. Highest electric impedance of the vagina day was defined as day EH in one estrous cycle, and the following 3 days as E2, E3, and E4. * Denotes statistical significance in the same group ($P<0.05$), and # denotes statistical significance between MS and NS in the same phase ($P<0.05$)
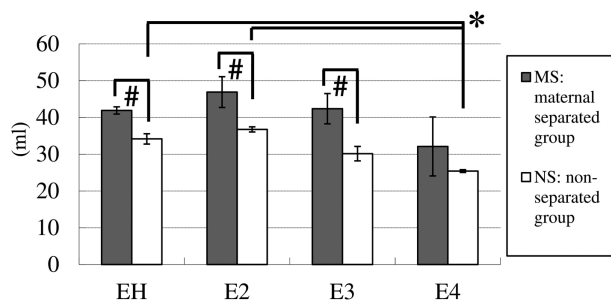

Fig. 3. Maternal separation and daily water consumption. Water consumption in each estrous phase of maternal separated (MS, gray column) and non-separated (NS, white column) female rats. Highest electric impedance of the vagina day was defined as day EH in one estrous cycle, and the following 3 days as E2, E3, and E4. * Denotes statistical significance in the same group ($P<0.05$), # denotes statistical significance between MS and NS in the same phase ($P<0.05$).

Discussion

In this study, we tried to determine the estrous cycle stage of female rats to investigate the relationship between eating disorder and stress. Measurement of vaginal electrical impedance was used for this purpose. Experiment 1 showed the highest EIV (day EH) mainly in the estrous phase. In our study, the association between vaginal impedance and vaginal smear differed from previous reports [1–3, 9, 33, 34]. Taradach reported that the peak EIV corresponded to the end of the proestrous phase [51]. This difference may have been due to the experimental conditions. The time of EIV

measurement between their study and our study was similar (14:00 − 16:00 h). In previous EIV studies, rats were maintained on regular light-dark cycles, but we maintained the rats on a reversed light-dark cycle in this study. The rats were manipulated for the stress study in their activity phase, which is their nocturnal phase, to avoid manipulation during their sleep. A 12-h separation between regular and reversed light-dark cycles made this phase difference which matched the highest EIV. Therefore we may have measured the declination phase of EIV. 89.7% of rats showed consistent association of EIV with their vaginal smear, measurement of vaginal impedance matched the estrous cycles in a reversed light-dark cycle. Therefore, we can define the highest EIV as the estrous phase in the dark cycle.

Based on the vaginal impedance study, the effects of estrous cycle on food and water intake were examined in MS and NS rats with estrous cycles matched by EIV measurement. MS rats showed stable food and water intake in the estrous cycle. NS rats showed decreased food and water intake on day E4 (this day matched the proestrous phase), while no significant changes in the other phases were observed. Our results showed that maternal separation changes feeding behavior in the estrous cycle later in life.

It is now widely accepted that the ovarian hormone estradiol plays an important role in the normal control of food intake in a variety of species [5, 6]. The ability of estradiol to influence food intake is best characterized in the female rat. Female rats show cyclic changes in eating during their estrous cycle, with reduced food intake occurring during the night of proestrus, following the rise of estradiol secretion that begins during diestrus and continuing into the afternoon of proestrus [5, 8, 53]. However, in our study, MS rats did not show this decrease in food intake. The pre-ovulatory increase in plasma estradiol concentration is associated with a transient decrease in food intake during estrus in cycling rats [10–13]. The phasic decrease in food intake during estrus is believed to be mediated by the pre-ovulatory increase in plasma estradiol concentration, and is attributed to the delayed action of estrogens peaking during the proestrus stage [13, 14, 19]. Experiments examining the effects of ovariectomy and hormone replacement have provided direct evidence that estradiol is the hormone responsible for changes in feeding behavior seen during the ovarian cycle [5, 12, 13, 19, 25, 52, 56]. The decrease in food intake that occurs during proestrus is accomplished by a decrease in meal size without a compensatory increase in meal frequency [5, 19]. Estrogen has been proposed to act directly and indirectly through orexigenic peptides such as ghrelin, or anorexigenic neuropeptides such as leptin [7, 45]. Fungfuang *et al.* reported that there was a significant increase in a serum leptin concentration and leptin mRNA expression in adipose tissue during the proestrous period compared with the diestrous period by using female Wistar-Imamichi rats aged 8–10 weeks [16]. And they also reported seven-weeks-old female Wistar-Imamichi ovariectomized rats, which were given 17β-estradiol benzoate showed higher serum leptin concentration and leptin mRNA expression than control rats [17]. It is suggested that anorexigenic agents such as leptin mediates the estrogen anorexigenic effect. Therefore, the history of maternal separation may impede the estrogen-leptin pathway or the activity of leptin.

On the other hands, MS rats drank more water than NS rats on the day EH, E1, and E2, and MS rats didn't show estrous cyclic changes of water drinking. There was a separation food intake from water drinking affected by maternal separation. Food intake and water drinking are traditionally seen as contemporaneous behaviors. However there are studies examined the independence of food intake and water drinking. Zorilla *et al.* examined of the intervals of water drinking during 24 h, *ad libitum* periods, and under these conditions, significant amounts of water are consumed independent of food intake [58]. Ghrelin administration, while stimulating food intake, inhibits angiotensin II–induced drinking, as well as drinking in response to hyperosmolar challenge [42]. Obestatin was originally identified as a posttranslational product of the ghrelin preprohromone [57]. Pretreatment with a selective obestatin antiserum resulted in a highly significant increase in water drinking in *ad libitum* fed and watered rats [50]. Food intake was also elevated in the antiserum-treated animals, although that increase failed to reach statistical significance. Based on these findings, one plausible explanation for this separation food intake from water drinking in our results is the effects of maternal separation on some agents inhibit water drinking independently or to be concomitant with food intake. Further studies are needed to clarify them.

Inoue *et al.* reported that female rats become hyperphagic after a time-restricted feeding schedule, and this rebound hyperphagia was further enhanced with additional psychological stress of space restriction [26, 27].

Furthermore, we found that female rats at 6 − 9 weeks of age with a history of maternal separation consumed a normal amount of food under standard feeding conditions, but consumed more food in rebound hyperphagia after a 2-h time-restricted scheduled feeding for 6 days [28]. This suggests that maternal separation disrupts responses to chronic hunger stress. Taken together, the results of this study indicate that maternal separation affects not only vulnerability of the estrous cycle itself, but ingestive behavior is affected by the estrous cycle. Results of this study indicate that maternal separation disturbs some functions of estradiol-linked feeding behavior. However, it is unclear which functions of estradiol are inhibited by maternal separation. To determine this association between maternal separation and eating behavior, further investigations are needed regarding meal size, estradiol concentration, administration of estradiol, ovarian histology, orexigenic and anorexigenic agents such as leptin, ghrelin, and obestatin.

This study has some limitations. Maternal separation, which is an integral stress, involves not only physical stress owing to lack of tactile stimulation, feeding restriction, and loss of protection, but also involves psychological stress. Therefore, it is unclear which aspect of maternal separation stress affects feeding behavior. Furthermore, because we used rats at 9 weeks of age, the effect of aging is unknown. And we avoided the stressful procedure, which is like collecting blood, in the experiment 2. However we should measure hormone levels, concentrations of orexigenic and anorexigenic agents and ovarian histology to determine the association between maternal separation and eating behavior, as mentioned above. Further investigation about ages and hormone levels is needed.

Conclusions

Vaginal electrical impedance showed the highest value in the estrous phase of rats housed in the reversed light-dark cycle. This method of measurement was quick, and useful for stress study in female rats. Using this technique, we found that maternal separation disturbs feeding behavior during the estrous cycle. This disturbance may be one way in which female-specific ingestive behavior may develop.

Conflict of Interest

The authors declare no conflict of interest associated with this manuscript.

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