Estrogen Increases c-Fos Expression in the Paraventricular Nucleus along with its Anorexic Effect in Developing Rats

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Abstract. Estrogen inhibits food intake in cycling females in a variety of species. To determine how the development of the anorexic system by estrogen is regulated, rat pups at four developmental stages, postnatal day 11 (P11)–13, P20–22, P25–27 and P29–31, and adult ovariectomized (OVX) rats received a daily subcutaneous injection of 20 μg/kg of estradiol benzoate (EB) or vehicle for three days. Food intake, body weight gain and immunohistochemical c-Fos expression in the brain were measured after each injection. EB treatment decreased both food intake and body weight gain from P27 onwards and significantly increased c-Fos expression in the parvicellular division of the paraventricular nucleus of the hypothalamus (pPVN), which is coincident with its anorexic effect in developing rats. The pattern of EB-induced c-Fos activation in other feeding-related nuclei did not coincide with its anorexic effect in developing pups. However, in adult OVX rats, EB treatment increased c-Fos expression in the nucleus tractus solitarius (NTS), the central nucleus of the amygdala (CeA), and, to a lesser degree, the ventromedial nucleus of the hypothalamus (VMH). These results suggested that the pPVN is an essential site in the brain for controlling the anorexic effect of estrogen and that the feeding system of rat begins to respond to estrogen before the onset of puberty (P25–28).

Key words: c-Fos, Development, Estrogen, Food intake, Hypothalamus

Understanding of the mechanism regulating eating is very important for human health because of obesity and eating disorders, such as anorexia and bulimia nervosa. Anorexia nervosa mainly occurs in young women around puberty, and the increasing estrogen level in the circulation during the puberty has been suggested to be involved in anorexia [1]. Estrogen exerts a potent physiological and inhibitory effect on feeding in a variety of species. During the menstrual cycle for example, meal size decreases during the periovulatory phase, just after the period of increased plasma estradiol (E2) concentration [2, 3]. In nonhuman primates like monkeys, food intake is reduced at the time of ovulation when estrogen is at its peak and increases after ovulation when progesterone is elevated [4]. In rats, food intake is decreased at proestrus when plasma E2 increases during the estrous cycle [5, 6], and ovariectomy increases food intake and body weight and E2 treatment normalizes these increases in ovariectomized (OVX) rats [7–9].

In rats, the anorexic effect of estrogen has been only reported to appear in 45-day-old rats ovariectomized either on the day of birth or at weaning [10, 11], and daily treatment of estradiol benzoate (EB) did not decrease food intake or body weight from postnatal day 30 (P30) until approximate P40 in OVX rats [12]. In contrast, the periodic alterations in food intake and meal size have been reported to occur prior to the vaginal opening in intact female rats [13], suggesting that the anorexic effect of estrogen appears prior to puberty in intact rats. Thus, the development of the anorexic effect of estrogen remains to be understood. In addition, recently, the technique using a meta-analysis of genome-wide association showed that determination of the critical period of the physiological phenomenon may lead to identification of genes related with this phenomenon [14]. Therefore, examining the critical period in the development of the anorexic action of estrogen under normal development is important to clarify the anorexic mechanism of estrogen.

The brain is an important site where estrogen acts and inhibit food intake [15]. Several areas within and outside of the hypothalamus have been reported to be involved in the estrogen action on feeding. For instance, E2 implants in the paraventricular nucleus (PVN) [16], the ventromedial nucleus of the hypothalamus (VMH) [17] and the arcuate nucleus (ARC) [18] lowered food intake and/or body weight in rats. Also, administration of EB onto the surface of the hindbrain over the caudal nucleus tractus solitarius (cNTS) decreased food intake [19]. In addition, E2 treatment increased feeding or cholecystokinin (CCK)-induced expression of c-Fos, a functional marker for mapping polysynaptic neuronal activity in the central nervous system, in several nuclei, such as the NTS, PVN and central nucleus of the amygdala (CeA) [20–22].

Estrogen affects functions of various parts of the brain directly and indirectly. This multiplicity makes it difficult to identify the specific brain area(s) related to the anorexic action of estrogen. However, during the gradual occurrence of development after birth, it could be considered that the appearance of c-Fos responsiveness to estrogen in the brain area(s) related to the anorexic effect of estrogen is highly correlated with the appearance of the anorexic action of estrogen. Therefore, in this study, to determine the critical period of the appearance of the anorexic effect of estrogen after birth and the brain area(s) related to this estrogen action, we investigated the developments of both changes of food intake and c-Fos expression of the brain after treatment with estrogen in intact
Materials and Methods

Animals

Adult Wistar rats (Charles River, Yokohama, Japan) weighing 180–210 g at the onset of the study were housed individually and maintained in a light (12:12-h light-dark cycle, lights on at 0600 h) and temperature (23 ± 1 °C) conditioned room. Females were timed-mated, and offspring in several different developing stages were used in the present study. The day of birth was considered postnatal day 0 (P0). On P1, litters were culled to 10 pups per mother (the number of females or males was 4–6). On P19, the pups were weaned and housed individually. All adult rats were provided with a pellet rat chow (Oriental Yeast, Tokyo, Japan) and ad libitum access to water unless otherwise noted. All animal procedures were approved by the Animal Committee at Fukui University and followed the ethical guidelines, as appropriate.

Effects of EB on food intake in developing pups

Alteration in the effect of estrogen on food intake was investigated in pups during four development stages: P11–14, P20–22, P25–27 and P29–31. Because male rats can also respond to estrogen in food intake [23], we investigated the response of both sexes of developing pups to exogenous estrogen. Thirty pups from three litters were provided for each of the four stages and divided into estrogen- (n=15) and vehicle-treated groups (n=15). All pups were subcutaneously (s.c.) injected daily with 20 μg/kg of estradiol benzoate (EB; Sigma, St. Louis, MO, USA) in 50 μl sesame oil (Nacalai Tesque, Kyoto, Japan) or the vehicle alone at 1200 h for three successive days. This dose of EB is enough to decrease food intake in adult rats [24, 25] and has no obvious side effect in P25–31 pups. The vagina did not open in the EB- and vehicle-treated rats in any of the present experimental groups.

Daily EB injection was started on P11, P20, P25 or P29 in each group and continued for three days. Before weaning, the pups were weighed and s.c. injected with EB or vehicle at 1200 h on P11, P12 and P13 and quickly returned to their mother. At 1200 h on P12, P13 and P14, body weight gain during 24 h was measured. After weaning, pups were deprived of food at 1200 h and s.c. injected with EB or vehicle (days of injection: P20–22, P25–27 and P29–31). The rodent pellet chow was replaced at 1800 h when the dark phase started, and a 2-h food intake was measured at 2000 h. On the next day, body weight gain and food intake for the preceding 18 h were measured at 1200 h (days of measurement: P21–22, P26–27 and P30–31) except for the last days (P23, P28 and P32), on which 24-h body weight gain and food intake were measured at 1800 h.

Effect of EB on c-Fos expression in the brain

To evaluate the change in histological pattern of neurons activated by estrogen, c-Fos expression was determined using three stages of postweaning pups (P20–22, P25–27 and P29–31). Fifty-four pups were randomly divided and were injected daily with EB (20 μg/kg s.c.) or vehicle for three days at 0900 h. The animals were allowed free access to food and water. Three hours after each injection (n=3, respectively), pups were deeply anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg, Dainippon Pharma, Osaka, Japan) and transected radially with 0.1 M phosphate buffered saline (PBS), pH 7.4, and then with 4% paraformaldehyde (PFA) containing picric acid in 0.1 M PBS, pH 7.4. The brains were removed immediately, postfixed in the same fixative overnight at 4 °C and cryoprotected in 30% sucrose at 4 °C.

Forebrain and hindbrain blocks were cut into coronal sections (40 μm) with a cryostat (Leica Microsystems, Nagoya, Japan). The forebrain and hindbrain blocks were from the optic chiasm to the median eminence and the entire NTS [26], respectively. The free-floating sections were stored at 4 °C until c-Fos immunohistochemical staining.

Adult female Wistar rats (n=5, 180–210 g at study onset) were ovariecomized bilaterally under diethyl ether (Nacalai Tesque) anesthesia. After recovery for one week, they were subjected to daily s.c. injections of EB (20 μg/kg in 100 μl sesame oil, n=3) or vehicle alone (100 μl, n=2) for three successive days at 0900 h. Three hours after the last administration, animals were deeply anesthetized with sodium pentobarbital (65 mg/kg i.p.), and the brains were collected with the same procedure described above.

Immunohistochemistry for c-Fos

Free-floating sections were washed in 0.01 M PBS, incubated for 30 min in 0.3% H2O2 in PBS at room temperature, blocked in 2% normal goat serum (Dako, Denmark A/S) solution in PBS for 2 h and incubated with rabbit polyclonal anti-c-Fos peptide antibody (1:20,000 in PBS; Ab-5, Calbiochem, Darmstadt, Germany) at 4 °C overnight. Sections were washed extensively in PBS containing 0.2% Triton X-100 (Nacalai Tesque; PBST) and incubated for 1 h with a biotinylated anti-rabbit goat IgG (1:1,000 in PBST; Vector Laboratories, Burlingame, CA, USA). Bound secondary antibody was amplified during a 30-min incubation of the sections in an avidin-biotin complex (1:62.5 in PBS; Vectorstain ABC Elite Kit, Vector Laboratories). Antibody complexes were visualized by immersing the tissues in 2% diaminobenzidine (Vector Laboratories). This reaction was stopped by rinsing the sections in 50 mM Tris-HCL (pH7.4). Sections were then mounted on gelatin-coated microscope slides and covered with a coverslip using Mount Quick (Daigo Sangyo, Japan). Labelling specificity was insured by omitting the primary or secondary antibodies.

Sections were inspected through a microscope (Olympus, BH-2, Tokyo, Japan). Cells were considered c-Fos-immunoreactive (ir) if they contained dark, punctate, nuclear immunostaining. The boundaries of the feeding-related nerve nuclei were delineated on the basis of anatomic landmarks (like fornix, optic tract and cerebral ventricles) and a rat brain atlas [26]. Although the brain size of the pups was smaller than that of the adults, the topographical landmarks of the various nuclei were similar to those of the adult brain as delineated in the rat brain atlas [26]. The following areas were examined as the feeding-related regions according to a previous report [27]: the parvocellular division of the paraventricular nucleus (pPVN), magnocellular division of the paraventricular nucleus (mPVN), arcuate nucleus (ARC), ventromedial nucleus (VMH), dorsomedial nucleus of the
hypothalamus (DMH), central nucleus of the amygdale (CeA), nucleus tractus solitarius (NTS) and area postrema (AP). The number of c-Fos-ir neurons in the pPVN (1.8–2.1 mm caudal to the bregma) was counted under a microscope at 400× magnification from four sections in each animal (n=3) for statistical analysis.

Statistical analysis

Data are presented as means ± SEM. Differences of daily body weight gain, food intake and water intake were compared between the EB- and vehicle-treated groups by an unpaired Student’s t-test using the StatMate III statistical software (ATMS, Tokyo, Japan). The numbers of c-Fos-ir cells in the pPVN on P22, P27 and P31 were analyzed by a two-way ANOVA (hormone treatment by postnatal day). When significant effects were detected by ANOVA (P<0.05), differences between individual means were examined with Tukey’s honestly significant difference test. P<0.05 was considered significant.

Results

Effects of EB on food intake in developing pups

Different groups of animals received daily s.c. injections of EB at 1200 h for three days starting on P11, P20, P25 or P29. In the group that started on P11, body weight was measured every day. A single daily injection of EB produced no effect on 24-h body weight gain for the three days (between P12–14) in either female or male pups (data not shown). Also, no significant effect of a single daily EB injection on body weight gain or food intake after 18 or 24 h from the onset of treatment from P20 to P22 was observed in either female (Fig. 1A) or male pups. When EB treatment started on P25, a significant decrease in both 18-h (or 24-h) body weight gain and food intake was observed on both P27 (second day) and P28 (third day), but not on P26 (first day) after the first injection in either female (Fig. 1B) or male pups. Also, EB treatment starting on P29 decreased both 18-h (or 24-h) body weight gain and food intake on both P31 (second day) and P32 (third day) significantly, but not on P30 (first day) in either female (Fig. 1C) or male pups. On the other hand, water intake was significantly decreased only on P31 and P32 by EB treatment starting on P29, but not on other days (data not shown). EB did not affect 2-h food and water intake after every injection in all developing pups tested.

Effect of EB on c-Fos expression in the brains

In the forebrain of vehicle-treated developing animals, there was robust c-Fos expression in the DMH, moderate expression in the CeA and VMH and a few scattered or no obvious expression in the ARC, pPVN and mPVN. Compared with vehicle-treated animals, there were intense (more than twice the amount) increases of c-Fos expression in the pPVN after the second injection (P26 and P30, Table 1) and third injections on P27 (Fig. 2B) and P31 (Fig. 2C). This is consistent with development of the anorexic effect of EB. However, no increase in c-Fos expression was observed in the pPVN on other days, P20–22 (Fig. 2A, Table 1), P25 and P29 (Table 1). In the mPVN, a moderate (less than twice or half the amount) increase and decrease were observed only on P26 and on P20 and P27, respectively (Table 1). In the CeA, a moderate increase and decrease were observed on P21 and P30 and on P27 and P29, respectively (Table 1). In the VMH, a moderate increase and decrease were observed only on P20–21 and P25 and on P26–27 and P30, respectively (Table 1). No obvious c-Fos expression was observed in the ARC of the EB-treated group as in the control group (Table 1). In the hindbrain, there was very little c-Fos expression in the AP and a few scattered c-Fos-ir cells in the NTS in the vehicle-treated control groups. No obvious c-Fos expression was observed in the AP of the EB-treated group as in the control group (Table 1). In the NTS, a moderate increase and decrease were observed on P25 and on P20, P22, P26 and P29, respectively (Table 1).

In the adult O VX rats treated with vehicle, there was robust c- Fos expression in the DMH, moderate expression in the CeA and the VMH, scattered expression in the pPVN and mPVN and no obvious expression in the ARC. An intense increase of c-Fos expression in the EB–treated group was observed only in the pPVN and was similar to that on P26–27 and P30–31 in the developing animals (Table 1). Moderate increases of c-Fos expression in the EB–treated group were observed in the CeA, VMH, NTS, but no obvious change was observed in the mPVN, ARC and AP (Table 1). In these areas in the developing animals, except for the pPVN, a moderate increase or decrease was observed in some EB-treated groups, but these changes were not consistent with those observed in the EB-treated O VX adult animals (Table 1).

The number of c-Fos-ir cells in the pPVN was counted on P22, P27 and P31 after the daily EB injections for three days. Two-way ANOVA showed that there was a significant difference between treatments, F(1, 67)=4.12 (P<0.05), while there was no significant difference in the interactive effect between treatment and age, F(2, 67)=2.19 (P>0.05), or among ages, F(2, 67)=2.61 (P>0.05). EB treatment was observed to significantly increase the number of c- Fos-ir cells in the pPVN on both P27 and P31 (P<0.01, Tukey’s honestly significant difference test), but not on P22, compared with the corresponding controls (Fig. 4). Furthermore, the increase in the number of c-Fos-ir cells in the pPVN on P31 in the EB-treated group was significantly greater than that on P27 (P<0.01, Fig. 4). Expression of c-Fos in the brain showed no obvious difference between the male and female pups in either the EB or control groups.

Discussion

In this study, we examined the effect of EB (20 μg/kg) treatment on both food intake and body weight gain in developing rats and demonstrated that estrogen treatment had no effect on food intake in early postnatal days, an observation that is consistent with previous reports [10–12]. However, our results indicate that the effect of estrogen treatment appear to become functional around P27 and onwards. This anorexic action of estrogen may have some physiological implications, since cell nuclear estrogen receptor binding in the brain reached adult level by P25–26 [28] and the plasma E2 level at P28 was close to adult level [29]. The anorexic effect of leptin [30] and expression of POMC and α-melanocyte stimulating hormone (α-MSH) [31] in rats also appeared during P21–28 as observed in adults. Thus, the anorexic neural system
including estrogen and leptin action seems to be developed and functional around P30, before maturation of reproductive function. Furthermore, EB only increased c-Fos expression in the pPVN in parallel with its anorexic effect in developing pups, while the changes in c-Fos expression after EB administration in several nuclei that have been reported to be involved in feeding behavior, such as the NTS, VMH, CeA, ARC, DMH and mPVN, were not concurrently observed with its anorexic effect in developing pups. These results suggest that the pPVN is a primary area in the brain for controlling the anorexic effect of estrogen.

The findings of the present study demonstrated that estrogen’s ability to modulate food intake is present at least from P27 onwards in intact rats. Wade and Zucker [12] had previously reported that EB did not decrease food intake until approximate P40 in rats ovariectomized either on the day of birth or at weaning. It is difficult to explain clearly the difference between their results and ours, but the most noticeable difference is the existence of the ovary. Thus, one possible explanation is that a gonadal factor(s) might be involved in the appearance of the anorexic effect of estrogen around P30 in female rats.

In male rats, the anorexic effect of estrogen appeared starting on P27, as was observed in the females. Two possibilities could be considered. One is that estrogen is a main factor to induce the development of the anorexic action of estrogen and that in male rats

![Fig. 1. Effect of EB injection on body weight gain and food intake in female pups. Female pups on P20 (A), P25 (B) and P29 (C) were daily s.c. injected daily with 20 μg/kg EB or 50 μl vehicle at 1200 h for three days. Data are shown as means ± SEM, and n = 7–8/treated group. Body weight gain and food intake after the first and second injections are for 18 h (P21–22, P26–27 and P30–31), while data after the third injection are for 24 h (P23, P28 and P32). The EB-treated group is significantly different from the corresponding vehicle-treated group (*, ** and ***: P<0.05, P<0.01 and P<0.001, respectively).]
EFFECT OF ESTROGEN IN DEVELOPING RATS

Testosterone is converted to estrogen by the aromatase in the brain [32]. The other is that the development of the anorexic effect of estrogen in both sexes is regulated by a mechanism that is independent of gonadal steroids or the gonad itself. If the latter is the case, verification of the estrogen action in the male rat brain may provide a promising approach for future clarification of estrogenic regulation of feeding behavior.

Our immunohistochemical data identified the estrogen-responsive site during development. Expression of c-Fos increased in response to estrogen treatment. Table 1 summarizes the effects of estrogen (EB) on c-Fos expression in the brains of developing pups and OVX adults.

Table 1. Effects of EB on c-Fos expression in the brains of developing pups and OVX adults

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- : No obvious change of c-Fos expression between the EB and control groups. ↓: Moderate (less than half) decrease of c-Fos expression in the EB-treated group compared with the control group. ↑ and ↑↑: Moderate (less than twice) and intense (more than twice) increase of c-Fos expression in the EB-treated group compared with the control group, respectively. Pups received a daily injection of EB or vehicle on the first (P20, P25 and P29), second, (P21, P26 and P30) and third day (P22, P27, P31; n=3/treated group/day) and were sacrificed 3 h after the first, second or third injection. Adult OVX rats were injected with EB or vehicle for three days and sacrificed 3 h after the third injection.

pPVN, parvocellular division of the paraventricular nucleus of the hypothalamus; mPVN, magnocellular division of the PVN; CeA, central nucleus of the amygdala; ARC, arcuate nucleus; VMH, ventromedial hypothalamus; AP, area postrema; NTS, nucleus of the solitary tract.

Fig. 2. Representative photomicrographs demonstrating c-Fos expression in the pPVN. Pups on P20 (A), P25 (B) and P29 (C) were s.c. injected daily with vehicle (left) or EB (right) for three days and sacrificed 3 h after the last injection on P22 (A), P27 (B) and P31 (C), respectively. The inset in C shows a larger magnification of the c-Fos expression. *: Third ventricle. Scale bars: 100 μm.

Fig. 3. Representative photomicrographs demonstrating c-Fos expression in the NTS. Pups on P29 (A) and adult OVX rats (B) were s.c. injected daily with vehicle (left) or EB (right) for three days and sacrificed 3 h after the last injection. Scale bars: 100 μm.
It showed an anorexic effect, indicating that a parallel relationship until P32, EB treatment did not increase c-Fos expression, although in the adult rats, in the NTS, CeA and VMH of the developing rats ized in the NTS, CeA and VMH [33, 34]. Contrary to the findings in the CeA both in feeding state and after CCK injection [20, 21]. E2 rats [17]. It has also been reported that estrogen receptor implants in the VMH lowered food intake and/or body weight in

Our results in adult rats showed that EB treatment decreased food intake and increased c-Fos expression in the pPVN, CeA and NTS and slightly increased the expression in the VMH. It should be noted that the changes of the pPVN were in parallel with the anorexic effect of EB. During P21 to P23, EB had no effect on either feeding or c-Fos expression in the pPVN. After P27, as well as in the adults, EB decreased food intake and increased c-Fos expression in the pPVN in a parallel manner. After the first injection, EB had no effect on either feeding or c-Fos expression in the pPVN, while after the second and third injections, EB affected both feeding and c-Fos expression in the pPVN. These results suggest the existence of a functional interaction between the neurons expressing c-Fos and the anorexic mechanism activated by estrogen. However, the delayed expression of c-Fos, an immediate-early gene, observed in the present study is difficult to explain based on our findings and will have to await further investigation.

We assume that the changes of the pPVN were in parallel with the anorexic effect of EB. During P21 to P23, EB had no effect on either feeding or c-Fos expression in the pPVN. After P27, as well as in the adults, EB decreased food intake and increased c-Fos expression in the pPVN in a parallel manner.

Fig. 4. Effect of EB injection on c-Fos expression in the pPVN. Pups on P19, P24 and P28 were s.c. injected daily with 20 μg/kg EB or 50 μl vehicle for three days and sacrificed 3 h after the last injection on P22, P27 and P31, respectively. Data are the numbers of c-Fos-ir cells per 40 μm section and are shown as means ± SEM. Four sections were observed in every brain (n=3/group/day). *: The number of c-Fos-ir cells in the EB group is different from the control group (P<0.01); +: The number of c-Fos-ir cells in the EB group on P27 is different from that on P22 (P<0.01). #: The number of c-Fos-ir cells in the EB group on P31 is different from that on P27 (P<0.01; two-way ANOVA with post hoc Tukey’s honestly significant difference test).

does not exist in the change between estrogen-induced anorexic action and c-Fos expression in developing rats. The occurrence of c-Fos-independent activation in the NTS, CeA and VMH cannot be excluded by the findings in this study, but it should be mentioned that the c-Fos-dependent system observed in the adult rats in the NTS, CeA and VMH was not yet developed on P31.

In the ARC, the increase of c-Fos expression after EB treatment was not detected in either developing or adult rats. Similar results have been reported in some studies. For example, there was no increase in c-Fos expression in the ARC 1.5 or 3 h after a single EB treatment [27] and 24 h after a single E2 administration [35] in OVX rats, although E2 increased c-Fos expression in POMC neurons in rats [36] and decreased it in NPY/AgRP neurons in fasted rats [37]. Furthermore, Peterfi et al. [35] reported that E2 administration tended to increase c-Fos expression 24 h after a single injection in rats, but the changes failed to reach the level of statistical significance. Thus, using c-Fos expression as a marker of neuron activation, it is difficult to detect the response of ARC neurons to estrogen treatment in rats, which is especially related to feeding regulation. The cyclic changes of feeding during the estrous cycle were abolished in mice lacking the AgRP/NPY neurons in the ARC [37], and both NPY/AgRP and POMC neurons have been reported to innervate to PVN neurons [38, 39], suggesting the interaction between the ARC and PVN in the anorexic effect of estrogen.

The previous reports about the estrogen action in the PVN are controversial. Butera reported that implantation of E2 into the PVN significantly decreased food intake in OVX adult rats [40, 41] and that PVN lesion blocked E2-induced suppression of food intake [42]. However, Hrupka et al. [43] reported that E2 implantation into the PVN failed to reduce feeding in OVX adult rats, and Dagnault and Richard [44] reported that PVN lesions did not prevent the anorexic effect of E2. Our results support Butera’s views. Dagnault and Richard [44] destroyed a smaller area of the pPVN compared with that reported by Butera et al. [42]. Our results showed that EB treatment induced c-Fos expression mainly in the ventromedial part of the pPVN in developing and adult rats. Therefore, one possible explanation is that the site of the estrogen implantation or the lesion in the PVN could account for the different results concerning the involvement of the PVN in estrogen action on food intake.

CRH antagonist reversed the eating-inhibitory effect of estrogen [45]. Oxytocin mRNA and immunoreactivity increased in the hypothalamus of OVX rats treated with E2 for more than 2 weeks [46, 47]. It has been also reported that intracerebroventricular administration of oxytocin and its agonist significantly decreased food intake in a dose-related manner in fasted rats [48]. Thus, CRH or oxytocin neurons in the PVN seem to be candidate mediators of the anorexic effect of estrogen. However, in the PVN of adult OVX rats that were injected daily with EB for three days, only 6% of c-Fos-ir neurons were CRH-ir, and there was no colocalization of c-Fos within oxytocin neurons (unpublished data). Therefore, other neurons appear to mediate the anorexic effect of estrogen, and possible candidates include, but are not limited to, α-MSH-positive and MC3/4 receptor-expressing neurons in the PVN [49, 50].
In conclusion, the present study demonstrated that EB (20 μg/kg, s.c.) administration increased c-Fos expression in the pPVN coincident with its anorexic effect in developing rats. It established a new method for studying the estrogen effect on feeding and suggested the pPVN as an essential site for control of the anorexic effect of estrogen. Furthermore, the current experiments proved that the feeding system of the rat begins to respond to estrogen before the onset of puberty (P25–28). Further studies about the significance of this response and neurons in the pPVN controlling the anorexic effect of estrogen might help to understand the neuronal networks of the estrogen effect in the brain, appetite control and eating disorders in women.

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