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Hormonal induction of lordosis and ear wiggling in rat pups: gender and age differences

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Abstract To assess how early can estrogens induce female mating behaviors, rat pups 8–29 days old (D8–D29, respectively) were injected twice daily with estradiol benzoate (E) or oil (O) followed by progesterone (P) or oil, and then observed for the estrogen-dependent ear wiggling (EW) and lordosis in response to natural stimulation from male rats. In female pups treated with E + E + P, the incidence of EW appeared as early as D13 and increased gradually to reach maximum at D18, when all pups tested showed EW. EW also occurred in E + E + O females, but never in O + O + P females or in any E + E + P male. Lordosis in E + E + P, as well as E + E + O, female pups occurred later, starting at D15. O + O + P females or E + E + P males never display lordosis. To explore the possibilities that the age and gender differences are due to distribution and/or function of estrogen receptor-α (ERα) or progesterone receptor (PR), separate pups were used for immunocytochemical (ICC) staining of these receptors in the hypothalamic ventromedial nucleus (VMN). There was no age difference in female pups in the density of ERα or the induction of PR between D11/D12, when no sexual behavior was observed, and D19/D20, when almost all pups tested performed the behaviors. There were gender differences: male pups had less ERα than females at D19/D20, though not at D11/D12, and did not respond to E in the induction of PR in the VMN. These results show that ERs and their signaling systems in the VMN of rat pups are functional at least after D11 but only in females, and that the gender differences appeared to be due to differences in the molecular biology of ERα.

Keywords Age difference · Ear wiggling · Estrogen · Estrogen receptor · Gender difference · Hypothalamic ventromedial nucleus · Immunocytochemistry · Lordosis · Progesterone receptor · Rat pup

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Introduction

Estrogens and progesterone act on the central nervous system, and one of their functions is to promote feminine sexual behavior—through induction, in part, by alterations of genomic functions by estrogens in the hypothalamus [1]. Estrogens bind to specific receptors, which in turn modulate gene expression. Such identified receptors acting as transcription factors are estrogen receptors (ER). In mice, ER and progesterone receptor (PR) genes are expressed prior to birth [2]. In rats, in both sexes, and as early as 24 h after birth, the ER density, measured from autoradiography, in various brain regions are close to adult levels. One exception is hypothalamic ventromedial nucleus (VMN), where ER density greatly increases from postnatal day 10 (D10) until adulthood [3]. The ER sex differences, with females having higher ER concentration than males, in the periventricular preoptic area (PVP), medial preoptic area (MPO), and bed nucleus of the stria terminus (BNST) were observed as early as D2 in pups and the magnitude is similar to that in adult rats; in VNM, ER sex difference is also present at D10 and later (D28–D49) [3].

Estrogen receptor is known to regulate PRs [4–7]. Also, the PRs that can be induced by estradiol are involved in female sexual receptivity [8]. In the VMN, the number of

PRs is increased through actions of estradiol; a minimum of 6 h of continuous exposure of estradiol is needed for the elevation of PRs in the cytoplasm and for the facilitation of lordosis behavior [9]. A number of studies, including Parsons et al. [5], showed that progesterone amplifies the effects of estrogens; it also activates proceptive behaviors such as ear wiggling (EW).

There are a number of studies showing the existence of ERs in rats before weaning, but to the best of our knowledge there were none to test the function of those receptors behaviorally. In the present study we used *naturally elicited estrogen-dependent* female sexual behaviors, lordosis, and EW to assess ER function. These behaviors were employed because their appearances indicate that ERs and their associated signaling systems and genomic actions are all functional. Earlier studies [10, 11] showed that rat pups could display lordosis as early as D6, but the behavior was not dependent on estrogen (estradiol benzoate) priming, and thus was not a manifestation of the functionality of ER. The aim of the present study is to determine the earliest age at which rat pups can perform female sexual behaviors, which are estrogen-dependent and are elicited naturally by male rats. Male and female rats were tested for sexual receptivity as early as D10 till D29. D10 was chosen mainly on the basis of our observations on general behaviors (see Results) and partly because, as mentioned above, VMN ER concentration increases from postnatal day 10. Also, injecting animals with estrogen at D10 does not interfere with the critical period for masculinization [12]. Thus, the animals (both males and females) were injected with an estrogen 48 and 24 h and with progesterone 4 h before testing. In order to ensure that sexual receptivity was due to estrogen priming, rats injected with oil instead of E served as control groups.

The measure of sexual receptivity was a lordosis reflex and proceptive behaviors, mainly EW. Lordosis reflex is a behavior used to study the effects of estrogens because it requires specific stimuli to trigger it and is only observable in rodents primed with estrogens [1]. EW is a proceptive behavior that occurs in response to priming with estrogens and progesterone.

Determining the earliest age at which ER are behaviorally functional is of great importance. The fact that ER are present, or even have binding capacity, does not necessarily mean that they are functional and thus able to induce sexual receptivity when occupied with their ligands. In contrast, behavioral study, which tests the outcome of interactions between ER and its ligands and the consequent activation of related signaling systems and genomic actions, can provide us with this information. Additionally, knowing the earliest age at which ER are functional has a practical significance. Animals used for electrophysiology studies are preferentially of young age. Knowing the earliest age that ER are functional would set age thresholds for animals that could be utilized for this purpose.

Methods

All procedures in handling and treating the animals were approved by The Rockefeller University's *Animal Care and Use Committee* in accordance with the Animal Welfare Act and the Department of Health and Human Services' "Guide for the Care and Use of Laboratory Animals".

Animal

Wistar rat pups with ages ranging from 8 to 29 days were used as subjects. The pups were bred in the laboratory. Prospective mothers were checked twice or more (around expected delivery day) daily. To test the sexual behaviors of the pups, a group of young adult males (B. Wt. 180–220 g) was maintained to provide 6–8 studs. In addition, 4–6 adult, ovariectomized females treated with estradiol (10 μg/day, sc, for two consecutive days before use) were employed to keep the male studs sexually active. All rats were housed at the Rockefeller University facilities with reversed light/dark cycles (lights on 9 pm through 9 am) under controlled temperature (24–25°C). Gender of the animal was determined on the basis of anogenital distance just before hormone treatment; rats with shorter distances were classified as females, and rats with longer distances were classified as males. This classification is reliable because the distances for male and female are well separated (usually about 2–3 mm) with no overlap. For example, at D8, the range of distances for males is 7.9–8.9 mm and that for females is 5.2–6.4 mm. Furthermore, gender was confirmed after behavioral testing, when animals were sacrificed. Each pup was used only once.

Hormonal treatments

There were three female and one male groups of animals. From each litter, pups are distributed as evenly as possible into at least two of these four groups so as to avoid genetic bias. Each of the three female groups received different treatments. In one group, female pups were subcutaneously injected with 17β-estradiol benzoate (E, 1 μg/0.1 ml/20 gm body weight) 48 and 24 h before behavioral tests, and with progesterone (P, 25 μg/0.05 ml/10 g body weight) 4–6 h before testing. The E dose is comparable to the lowest dose (0.1 μg/10 g body weight, one injection) given to rat pups by Williams [10]. These pups will be referred to as E + E + P females ($n = 164$, all age

groups). The second or E + E + O group ($n = 38$) consisted of pups that were injected with E 48 and 24 h and then with oil (O) 4–6 h before testing. Females in the third or O + O + P group ($n = 51$) were injected 48, 24, and 4 h with oil, oil, and progesterone, respectively. Male pups ($n = 54$) were subjected to only one, E + E + P, treatment. In all groups, a long hypodermic needle was used for injections in order to ensure that there was no leakage at the injection site; the needle was slowly withdrawn after injection.

Behavioral tests

Before the age of 8 days or D8, pups were observed undisturbed for ear detachment, eye opening, and general behaviors. Starting at D10, they were tested for the proceptive behavior, EW (with pups standing still and the vision of ears became blurred from fast movement), and receptive behavior, lordosis, in several ways under two experimental conditions. Younger pups, D10–D12, were tested in a warm and humid environment (testing cage in a water bath) to prevent heat loss so as to maximize the chance of observing the behaviors. They were first tested with stud males that were introduced one at a time into the testing cage. A stud was replaced if it showed no action (chasing, pushing, and/or licking) toward the pup for 2 min. Before the test, the studs were activated by introducing a receptive "lure" female into a stud's cage until the stud performed two mounts. After using up all stud males (up to 8, to maximize the chance of eliciting sexual behaviors), they were further subjected to: (1) stimulation by placing a male rat on their back and rump, (2) manual stimulation by an experimenter, and (3) stimulation by brushing their back, rump, and genital area with a big brush for 5 s.

Older pups were tested in the room where the animals were housed. Subjects were individually introduced to the male stud's home cages (one stud per cage), where the males were previously activated with receptive "lure" females. Each subject was observed for 20 min with males. To facilitate the test, if the stud did not perform any mount for 2 min, the subject was moved to the cage of another sexually active stud. If a pup was not mounted during the 20 min observation, a stud male, held by an experimenter, was placed on the pup's back 5 times, each for approximately 3 s.

Receptivity was assessed with lordosis strength (LS) and lordosis quotient (LQ). LS was scored with a 0–3 scale, with 3 being the strongest, when both the head and the rump were raised and chest depressed (see Fig. 5b), 1 the weak lordosis with body straighten and tailbase raised (e.g., Fig. 5a), and a 2 in between (Fig. 5d). Each lordosis is scored individually. For result presentation, the scores from all lordoses from all pups in a test group were averaged. All lordoses, regardless of the strength, were used to calculate LQ as (number of lordosis/number of mount) × 100. For the pups who responded, one placement of a male on the back is counted as a mount.

The testing took place during the dark phase of the light:dark cycle and 4–6 h after the last progesterone/oil injection. In addition to the experimenter's observation during the test, each test was also videotaped and viewed by another observer under a "blind" condition to score lordosis and EW independently. Each subject was tested only once.

Immunocytochemistry (ICC)

Separate groups of 32 male and 34 female pups were used for ER and PR ICC. For ER ICC, the pups were *not* treated with steroids so as to avoid the possibility of down- or up-regulating ER. They were perfused at the ages of D11/D12 (6 females and 10 males) and D19/D20 (16 each, male and female) first with 0.1% heparin in PBS and then 4% paraformaldehyde. Following perfusion, brains were removed and post-fixed in 4% paraformaldehyde overnight. Next, brains were soaked in 30% sucrose solution for 24 h or longer. Then, the brains were sectioned with a microtome; each section was 30 microns thick. The sections of VMN were collected in wells and washed in 0.1 M phosphate buffer saline (PBS); each wash lasted for 5 min. All the washing and the later ICC procedures were carried out with free floating sections in the wells. Next, they were placed in a solution containing hydrogen peroxide and PBS (100 µl of 30% H_2O_2 in 10 ml of PBS), and then washed in PBS (2 × 5 min).

After washing, the blocking step was carried out; sections were placed in a solution containing normal goat serum (NGS), Triton, and PBS (3 drops of NGS, 400 µl of Triton, 10 ml of PBS). Subsequently, sections were placed in a primary antibody against estrogen receptor alpha, (ERα polyclonal, 1:25000; UPSTATE), and incubated in a cold room (4°C) for 48 h. Then, they were washed in PBS (2 × 5 min) and exposed for 1 h to a secondary antibody (ABC elite kit from VECTOR), which consisted of 3 drops of NGS, 1 drop of IgG, and 10 ml of PBS. After sections were exposed to the secondary antibody, they were washed in PBS (4 × 5 min), and then treated with Vectastain ABC reagent (from VECTOR; 4 drops of reagent A and 4 drops of reagent B in 10 ml of PBS) for 1 h. After treatment in Vectastain ABC reagent, they were washed in PBS (2 × 5 min) and in Triz (1 × 5 min) (7.45 g of Trizma pre-set crystals in 1 l of distilled water). Consequently, the sections were stained in DAB (0.1 g of DAB, 200 ml of

0.05 M Triz; 0.2 ml of 3% hydrogen peroxide from Sigma); they were stained enough for the cells to be visible, and then the reaction was stopped by placing it in Triz solution. Next, the sections were mounted on superfrost plus slides and dried for 24 h. When the sections were dried, the slides were washed in distilled water, and then dehydrated in alcohols (70%, 95%, and 100% consecutively), washed in xylene, and cover slipped.

For PR ICC, the protocol described above for ERα was used with the following exceptions: PR antibody (Anti-Human PR from DakoCytomation, Carpinteria, CA, 1:500 dilution) was used as the primary antibody and tissue sections were post-fixed for 24 h after sectioning. A total of 12 female and 6 male pups were used for PR ICC. Unlike those used for ER ICC, each pup, except for the O + O controls, used for PR ICC was given two injections of E (0.1 mg/20 g body weight) 24 and 48 h before sacrifice in an attempt to induce PR.

The densities of ERα-immunoreactive (ER-ir) and PR-ir cells in VMN were counted with SCION program. A preselected circle (#110) was placed on the part of ventromedial hypothalamus where the density of the labeled cell was the highest, and the number of the labeled cells counted. To exclude background staining, the circle was placed on the cerebral cortex in the same section and the labeled cells, if any, were counted and subtracted from those in the VMN on the same side. The results are presented in two ways: the Average and the Highest density. In the Average, the mean density (number of net labeled cells/circle) of the two VMNs in the same tissue section was obtained and the average of the mean from all sections was calculated for each brain. For the Highest density, the number of the labeled cells from the VMN containing the highest densities in each of the brains was used to calculate group average.

Statistics

Numerical results are presented as 'Mean ± SEM (n)'. Comparisons were made by Chi-square or two-way ANOVA followed post hoc by Tukey test (Statistica, by StatSoft, Tulsa, Oklahoma). Differences are considered significant when $P < 0.05$.

Results

General observations

Starting at postnatal day 6 (D6), each pup was observed for eye opening, ear detachment, and movements. The results are summarized in Fig. 1. Ear detachment, which would allow for showing EW, was not an all-or-none phenomenon; the detachment was halfway at D10 and not complete until D12. Even then, the ears were still close to the head, as opposed to standing up as in adults. Therefore, the earliest possible age any EW can be observed is D10.

Pups started to walk, instead of crawling, on D10. They could walk upright, but with belly touching the floor and hind legs being dragged along during walking. Pups do not walk surely until D12 or D13. They were not moving actively even on D13; that is, they did not move around but tended to stay in one place if undisturbed. In order to exhibit lordosis posture, a pup should be capable of raising the head, the rump, and the tail; a pup that can only lie on the floor and cannot stand on four legs is unlikely to have the strength to do so. Thus, the earliest possible age to exhibit lordosis in response to stimulation by males is, again, D10, although non-mating, artificial stimulation may elicit lordosis on D6 [10]. This age, D10, was therefore chosen as the earliest age for behavioral tests.

Eye opening was first seen in some pups on D13 when EW was beginning to be observed in some female pups. Whether there is a link between the two events is not known. No gender difference in the time course of ear detachment, walking, or eye opening was observed.

Sexual behaviors

Ear wiggling can be induced in female but not male pups with E, with or without P, but not P alone

Results of EW observation are summarized in Fig. 2. In female E + E + P group ($n = 143$, all ages), EW was not seen until D13; none of the 25 pups from 11 litters (or 0/25/11) showed EW on D10–D12. On D13, EW was observed in 18% or 2 of the 11 pups from 3 litters (2/11/3). From

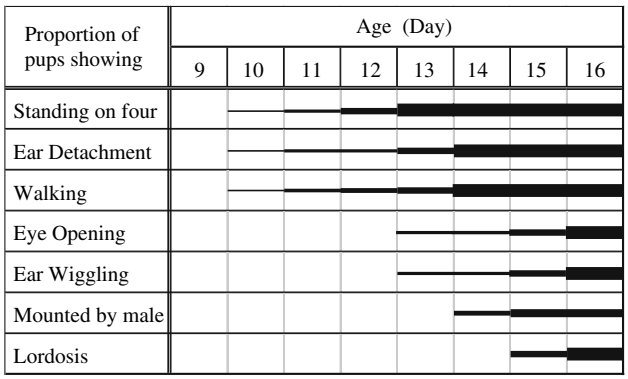

Fig. 1 Schematic summarization of general observations. The thickness of horizontal bars indicates the proportion of the pups displaying the observed trait at different ages (the thickest = 100%). Note that EW and lordosis occurred after ear detachment and standing by 3 and 5 days, respectively

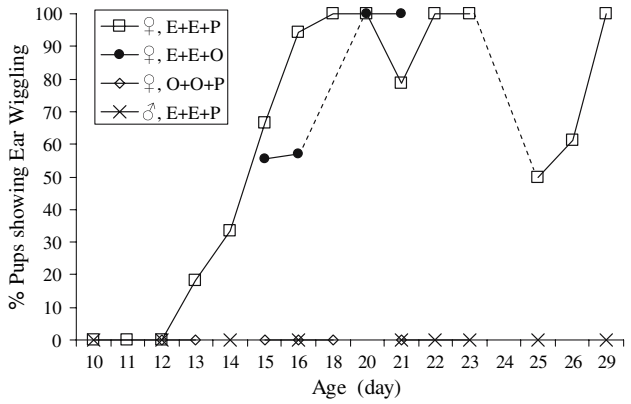

Fig. 2 Percentage of pups showing EW at various ages and with various treatments (E, estradiol benzoate; P, progesterone and O, oil). Note that E + E + P and E + E + O have very little difference, and that male pups with E + E + P and females with O + O + P did not display any EW. $N = 3$–24 for 32 out of 34 data points. The remaining two points (male at D12 and D23) $n = 1$. The dashed lines were used to connecting data points across gaps

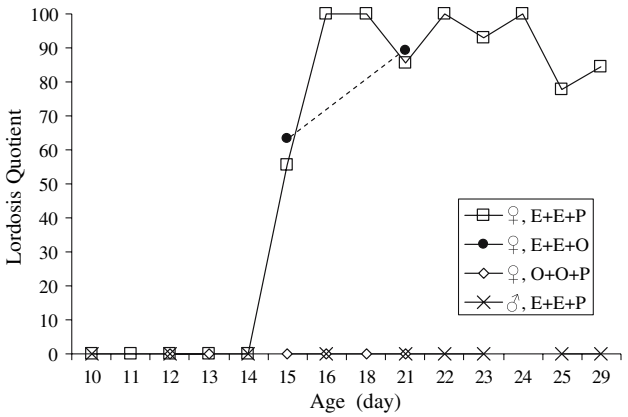

Fig. 3 Summary of observation on lordosis. Lordosis Quotient for each treatment group at different ages was calculated from cumulated lordoses and mounts from all the pups in the group. The results are similar to those of EW, except that lordosis occurred two days later on D15. $N = 3$–20 for 28 out 31 data points with the remaining three points (male at D12, 14, and 23) being 1 or 2

then on the proportion of pups showing EW increased steadily to reach maximal level by D18 (Fig. 2). From D18 to D29, although there were some fluctuations, the overall proportion of pups showing EW remained high (81% or 51/63/15). Female pups in E + E + O group also showed EW. The proportion is not statistically (Chi-square test, $P > 0.05$) different from that in E + E + P group on D15 (55.5% or 10/18/7), D18 (100% or 3/3/1), or D21 (100% or 6/6/2), although the level on D16 (57% or 4/7/4) is lower (Chi-square test, $P < 0.05$ (Fig. 2)). In contrast, none of the O + O + P female (0/51 pups/20 litters) or E + E + P male pups displayed EW (0/54 pups) (Fig. 2).

Lordosis, like EW, can be induced in female but not male pups with E, with or without P, but not P alone

Results for LQ and LS are summarized in Figs. 3 and 4, respectively. Lordosis in female pups can be elicited by a male's mounting (Fig. 5a and b) or by placing a male on the subject (Fig. 5c and d). One difficulty in testing lordosis is that male studs did not mount the pups as readily or frequently as they did to adult females. No mounting was ever observed before D14; none of the 31 pups observed on D10–D13 was mounted, regardless of treatments and gender, and despite the use of multiple studs. From D14 on, the number of pups mounted/number of pups observed are 55/98 (56%) for E + E + P females, 13/19 (68%) for E + E + O females, 11/42 (26%) for O + O + P females, and 15/42 (36%) for E + E + P males. Placing a stud on the back of a pup is less effective than mounting in triggering lordosis. It did so in only three female pups, one on D15 and two on D21. For these three pups, each placing

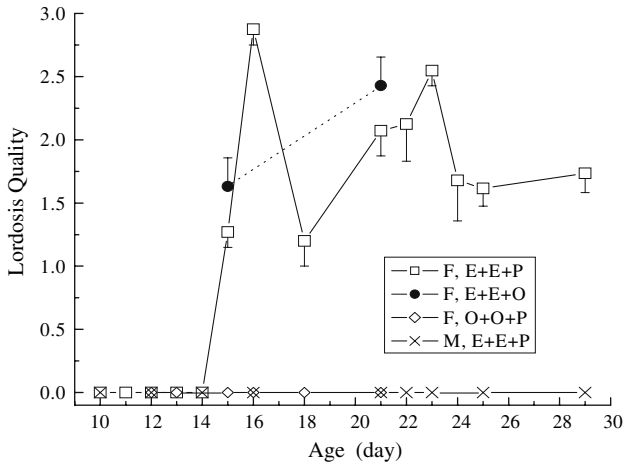

Fig. 4 Summary of observation on lordosis quality. The quality was scored with a 0–3 scale, with 3 being the strongest. Vertical bars are SEM. Ns for each of the data points are the same as in Fig. 3

was counted as one mount in calculating LQ. In female E + E + P group (129 pups over 14 age, see Fig. 3 for detail), lordosis first appeared on D15 (Figs. 3 and 5a) and LQ quickly reached and stayed on or near the maximal level from D16 on. Pups treated with E alone (E + E + O group) were tested on D15 ($n = 9$) and D21 ($n = 10$), and in both cases the results are statistically the same as in E + E + P group [Fig. 3, E + E + P vs. E + E + O and D15 vs. D21, two-way ANOVA, $F(1,25) = 0.0079$; $P = 0.93$], indicating that P was not essential. Like EW, lordosis was never observed in O + O + P females (51 pups over 6 age, Fig. 3) or E + E + P males (54 pups over 10 age, Fig. 3) either in response to mounting by studs or to placing a male on subject's back. Observations from all

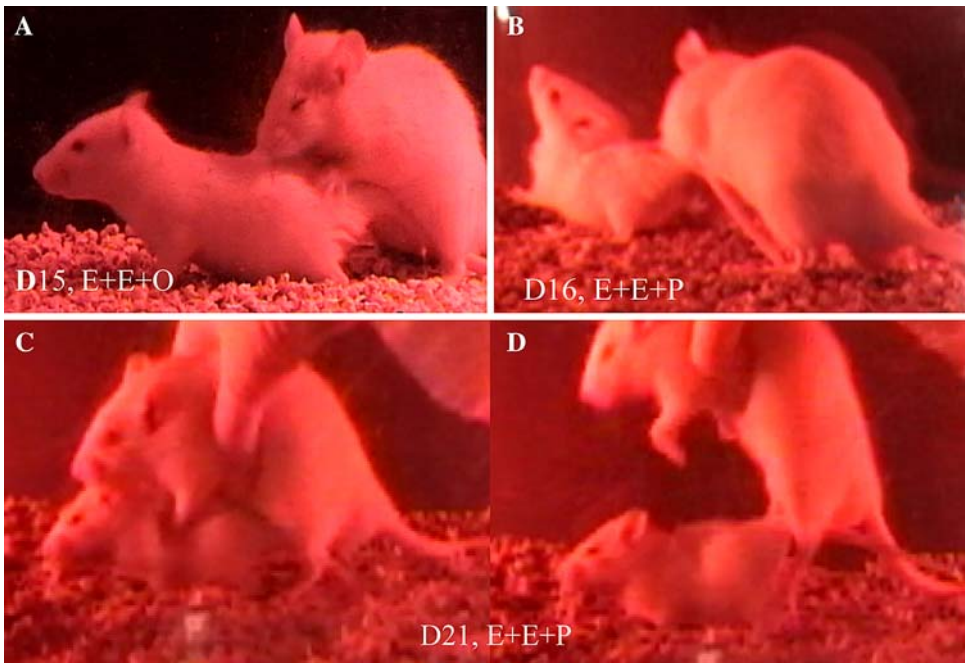

Fig. 5 Examples of lordosis. Lordosis could be elicited by a male rat mounting female pups as young as 15 days old (D15, **a**); by D16 they could exhibit strong lordosis (**b**). *Note*: both pups **a** and **b** also showed ear wiggling (blurred images of ears). Lordosis could also be elicited by placing a male on a female pup: (**c**) lordosis was elicited by placing the male on the pup and (**d**) the strong lordosis was still maintained after lifting the male. Ages and treatments of the subject pups are indicated

four groups together indicate that, as in adults, lordosis is estrogen-dependent and that there is a gender difference.

The strength or the quality of lordosis was about half-maximal when it first appeared on D15 (Fig. 5a) and reached maximal on D16 (Figs. 4 and 5b), but then fluctuated somewhat below the maximal level (Fig. 4). Results of E + E + O group are, again, essentially the same as those of E + E + P group [for treatment, $F(1,21) = 3.20$, $P = 0.087$, 2-way ANOVA].

Immunocytochemistry (ICC)

The density of estrogen receptor α-immunoreactive (ERα-ir) cells in the VMN appeared to be age- and gender-dependent

The densities of ERα-ir cells in the VMN of female and male pups are summarized in Table 1. In females, as expected, high density of ERα-ir cells was found in VMN and the arcuate on D11/12 and D19/20 (Fig. 6a and c). There is a marginal difference statistically between the ages; the difference is not significant when sampled as an Average but is significant as Highest (Table 1). High density of ERα-ir cells was also observed in the VMN and the arcuate of males on D11/12, but not on D19/20 (Fig. 6b and d). The densities for males on D11 are significantly ($P < 0.001$, 2-way ANOVA/Tukey) higher than those on D19/20 with both Average and Highest samplings. Comparisons between female and male show that there is no gender difference at the younger age (D11/12). But at D19/20, females have significantly ($P < 0.001$, 2-way ANOVA/Tukey) higher ERα-ir cells than males (Table 1).

Gender but not age difference in the density of PR-ir cells in the VMN

The results of PR-ir density in VMN are summarized in Table 2. PR-ir cells were detected only in E + E treated female D11 and D20 pups (Fig. 7a and c). When only the highest density of PR-ir from one VMN in each pup is

Table 1 Comparisons of estrogen receptor alpha densities in the VMN between genders and ages

Age (day)	Sampling category	Gender		*P* (ANOVA, Tukey)
		Female	Male	
11–12	Average	32.13 ± 2.75 (6)	38.12 ± 2.42 (10)	ns
	Highest	49.67 ± 5.87 (6)	45.70 ± 2.44 (10)	ns
19–20	Average	25.65 ± 1.46 (16)	10.24 ± 3.11 (16)	<0.001
	Highest	33.69 ± 1.81 (16)	12.25 ± 4.21 (16)	<0.001
P (ANOVA, Tukey)	Average	ns	<0.001	
	Highest	<0.05	<0.001	

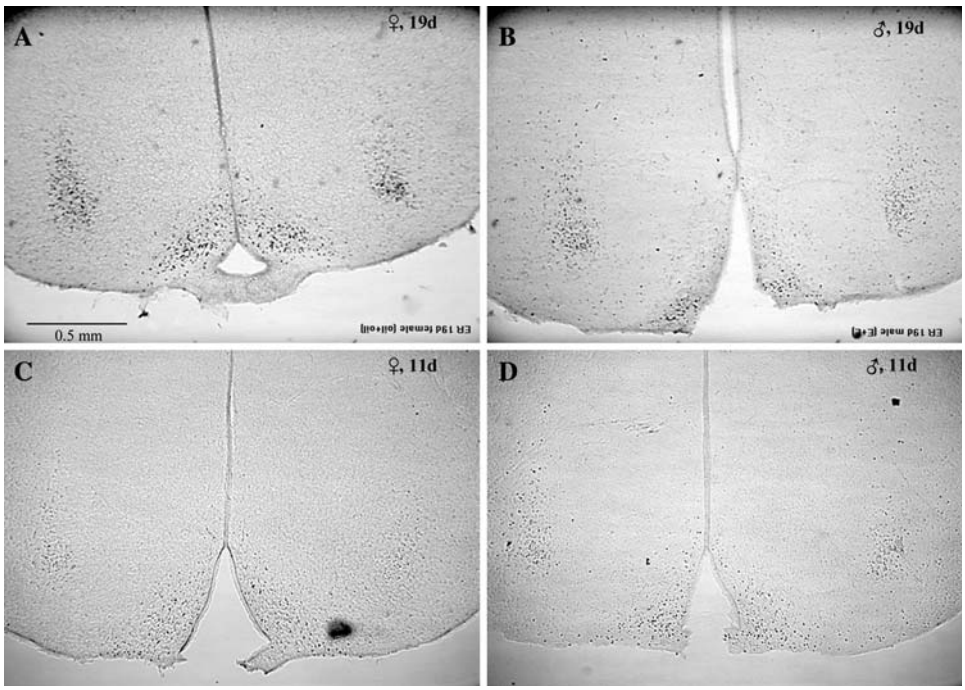

Fig. 6 Examples showing the ERα-ir cells in the ventromedial hypothalamus. There is no difference in the density and distribution of these cells between female pups with whom E could (**a**, D19) or could not (**c**, D11) induce lordosis. Thus, based on these results and those in Table 1, the density and the distribution of ERα-ir cells in the VMN alone cannot account for the age differences in behavioral response to E. Males have same density of ERα-ir cells as females at D11 (**d**), but males have less at D19 (**b**). Calibration bar: 0.35 mm for **a** and **b**; 0.5 mm for **c** and **d**

Table 2 Density of progesterone receptor (PR) in lateral VMN

Rat		Treatment	Sampling category	Density of PR-ir cells in lateral VMN, M ± SEM (*n*)	
Gender	Age (day)			Density	*P*, ANOVA
Female	11	E + E	Highest	53.00 ± 3.02 (4)	<0.02
Female	20	E + E	Highest	37.25 ± 2.63 (4)	
Female	11	E + E	Average	32.83 ± 2.23 (4)	ns
Female	20	E + E	Average	24.67 ± 3.48 (4)	
Female	20	Oil + Oil	Both	0 ± 0 (4)	
Male	20	E + E	Both	0 ± 0 (6)	

considered (Highest sampling), the average for D11 (53 ± 3 cell/unit area, $n = 4$) is significantly ($P < 0.02$, ANOVA) greater than that for D20 pups (37.3 ± 2.6 cell/unit area, $n = 4$). However, if the mean densities from each tissue section containing VMN are averaged (Average sampling), there is no significant difference between D11 (32.8 ± 2.2, $n = 4$) and D20 (24.7 ± 3.5, $n = 4$) pups. In contrast, in control females treated with O + O ($n = 4$), no PR-ir cell was observed in VMN or the arcuate (Fig. 7d). This contrast indicates that estrogens are required for the induction of PR. In E + E males ($n = 6$), PR-ir cell was not seen in the VMN. However, some were observed in the arcuate (Fig. 7b), indicating that the absence in the VMN was not due to a technical failure.

Discussion

In this study, we found that female rat pups as young as D13 could exhibit sexual behaviors in response to natural stimulation from males. However, there were three conditions to be met. First, the pups have to be primed with estrogens; progesterone is not necessary. Second, there is a gender difference: only female pups can be induced by estrogens to exhibit sexual behaviors; male pups do not show such behaviors in spite of hormonal treatment. Third, there is an age effect: sexual behavior could be observed only from D13 on, and not before. There are also corresponding gender differences in the function of ER in the VMN: at D19/20, when they can show lordosis, females have higher ER density than males, and PR can only be induced in female and not male pups.

Estrogen-dependence of the sexual behaviors exhibited by pups

In the present study, pups could perform female sexual behaviors only when they were treated with estradiol,

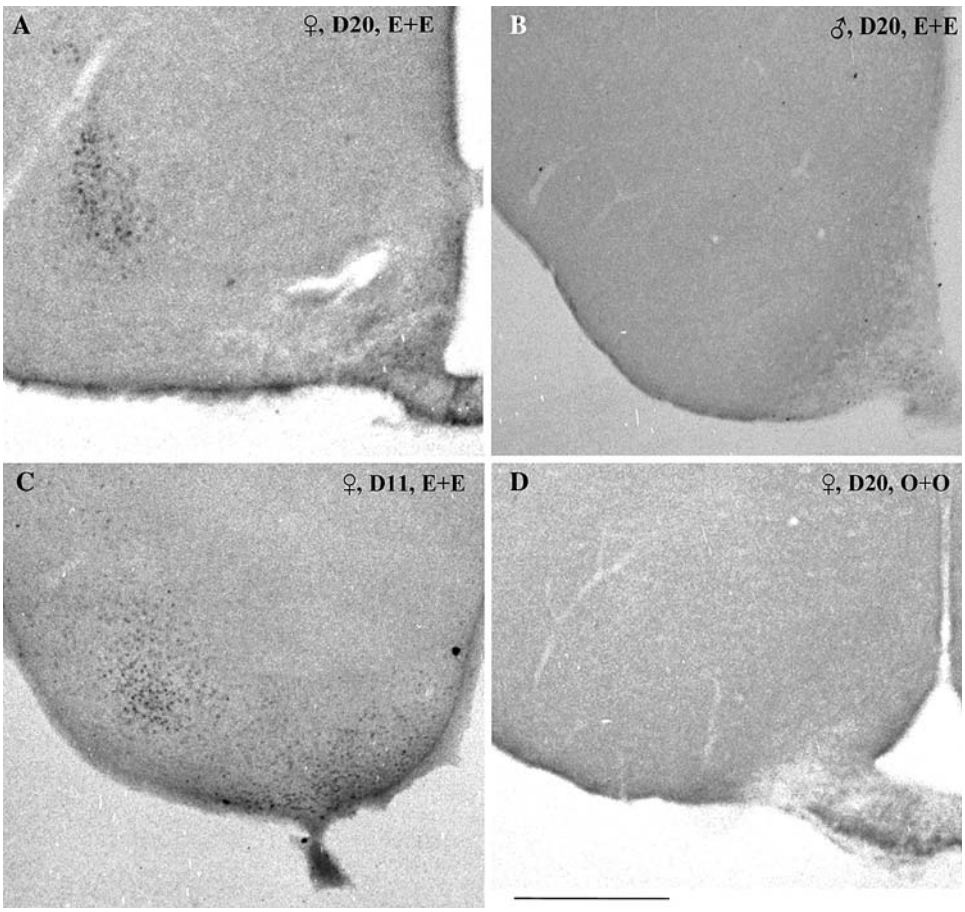

Fig. 7 Representative sections processed for PR ICC. (**a** and **c**) From females treated with E + E, PR-ir cells can be found in both the VMN and ARC, with a distribution pattern similar to that of ERα-ir cells, indicating that ERα, at least some of them, in females can mediate the induction of PR. (**b**) Males treated with E + E have PR-ir cells in the ARC but not in the VMN. This contrast raises the possibility that some ERs (in ARC) can mediate the induction of PR but others (in VMN) cannot. (**d**) A section from an O + O treated female did not have PR-ir cells in ARC or VMN. Calibration bar: 0.25 mm

indicating that, as is the case of adults, these behaviors are estrogen-dependent. In an earlier study [11], 6-day-old male and female infant rats treated only with oil were found to be capable of showing "EW" (head rotations presumably associated with EW) and "lordosis" (a posture resembling lordosis in response to an artificial stimulation). If the "lordosis" and "EW" observed in the earlier study were indeed sexual behaviors, then the earlier finding would indicate that at the infancy the sexual behaviors are *not* estrogen-dependent. This is in contrast to the present finding and the simplest explanation is the age difference. Another possibility is treatment difference. The infants in the earlier study were given oil only, while the control female pups in the present report received O + O + P treatment. It is possible that the pups failed to show sexual behaviors because they were abolished by the addition of P. However, this possibility is very remote because in the present study it was found: (i) E + E + P group was not worse than E + E + O (Figs. 2–4), and (ii) the presence of PR itself was estrogen-dependent (Fig. 7d) and without estrogen there would be no PR to mediate P effects of the O + O + P treatment.

The presence of ERs in the hypothalamus and other brain regions of young rats have long been demonstrated [13, 14]. However, the physical presence of ERs does not guarantee that estrogens can achieve their biological effects (see below in discussion of gender difference). One goal of the present study is to find out whether there are functional ERs, at least in terms of mediating estrogen-dependent female sexual behaviors, in the brain of pups, and if positive, how young. The exhibition of sexual behaviors, at least lordosis, requires the genomic actions of estrogens mediated through ERs in the brain, mainly the hypothalamus [15]. Therefore, the fact that female pups as young as D13 can show estrogen dependence of sexual behaviors indicates that pups at D11 or latest D12 (when they were primed with estrogens) not only have ERs in their brain but also that the ERs and their related signaling systems are also functional. Thus, female pups as young as D11 can be used as subjects for mechanistic analyses of behaviorally relevant estrogen effects.

Age effects in receptive behaviors

Female pups treated with estrogens started to show EW on D13 and lordosis on D15. Despite extra efforts, none of the receptive behaviors was observed before the respective

ages. These age differences do not appear to be due to a lack of functioning ER. ERs can be detected in embryos [2]. In the present study, ERs were visualized immunologically at D11. In fact, the density of ER in VMN appeared to be higher at D11/12 than at D19/20. Furthermore, PR-ir cells were observed in the VMN of D11 female pups primed with estrogens on D9 and D10. It is obvious from this observation that female pups not only have ERs in their hypothalamus but also that the ERs are functional. Therefore, it is very unlikely that the age differences in performing sexual behaviors are due to a difference in the presence or the functioning of ER.

Alternatively, the age differences in the behaviors may be due to insufficient motor capability. Before D13, when EW was first observed, only some pups had their ears detached from the head and these detachments were mostly incomplete. This may be a reflection that the development of motor control over the ears is not mature prior to D13. Similarly, although pups could walk before D15, their gaits were not normal. They might not have sufficient strength to exhibit lordosis, a demanding posture for motor control. EW and lordosis are motor activities and require complete neural pathways. It could be that the pathways for EW and lordosis are not fully developed until D13 and D15, respectively.

Another possibility is the lack of appropriate sensory inputs. Prior to D14, rat pups regardless of gender and treatments had never been observed to be mounted by male studs, which is the natural and most effective stimulation in eliciting female sexual behaviors. Therefore, they never received appropriate and sufficient stimulation, and this lack of sensory input may contribute to the failure of exhibiting sexual behavior, at least the lordosis. Although they have been stimulated by studs placed on their back, the stimulation might not be sufficient. Thus, whether ERs are functional or not prior to D9 is still an open question. But from the present findings it is certain that ERs are functional as early as D9.

Gender differences

One prominent finding in the present study is that while female pups could be induced by estrogens to show sexual behaviors, males could not. This is not due to physical differences as there was no sex difference in the time course of ear detachment, eye opening, standing, or walking. It is not because males are slow in development because they still did not exhibit sexual behaviors even at D29, when females have long reached the behavioral plateau. We then hypothesized that the variation in the presence and/or the functioning of ERs in the hypothalamus accounts for the behavioral differences.

With immunocytochemical (ICC) techniques, ERs were found not only in the VMN of female but also in that of male pups. In fact, the densities and, hence, the numbers of ERα-ir cells are the same statistically in male and female at D11/12, when E application could induce EW in females but not males. At D19/20, males have less ERα-ir cells than females. On the basis of this, one could predict that there would be graded, but not the observed all-or-none, differences in estrogen-dependent EW and lordosis. Obviously, the complete failure of male pups to show estrogen-dependent behaviors is not due to a lack of ER in VMN alone.

The above conclusion raises the possibility that ERs in male pups are not functional. This possibility is evaluated by examining PR induction. The induction of PR in the VMN is a well-established estrogen function mediated by ERs [16, 17]. This was confirmed for pups by our findings that PR was observed only in female pups treated with E + E but not in O + O treated control females. In male pups, E + E treatment could induce PR in the arcuate but not in VMN, indicating that the ERs in males' VMN are not functional.

The failure of PR induction per se cannot completely account for the failure of male pups to show receptive behaviors. In the present behavior study, there was practically no difference between E + E + P and E + E + O treatments in the induction of EW and lordosis in female pups. Progesterone, therefore, was not essential and has little contribution to the hormonal induction of receptive behaviors. Consequently, the presence or absence of PR should not make a difference. Thus, the failure of the induction of PR is a manifestation of ER being not functional but is not by itself the cause of the behavioral deficit.

Our ICC findings of gender differences in PR induction are in tune with our behavioral results. However, ours are not consistent with others' findings in that, in mice up to D8, males have more PR in the VMN than females [18]. The reasons for this are unknown other than the possible species difference.

There are, of course, many reasons that ER could be rendered non-functional. There may be some subtle structural differences between ERs in male versus female in VMN neurons. Secondly, there may be differences in the concentrations or locations of certain cofactors in male versus female. As well, there may be differences in the availability of functional signaling systems or in the ER coupling to signaling systems. There could also be problems of the expression of PR, alterations in the chromatin covering the PR gene promoter, in male VMN. Thus, finding out the causes underlying the gender differences observed in the present study will shed light on both ER and PR molecular biology as they relate to female reproductive behaviors.

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