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Estradiol Treatment during Perinatal Development Alters Adult Partner Preference, Mating Behavior and Estrogen Receptors α and β in the Female Mandarin Vole (*Microtus mandarinus*)

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During development, many aspects of behavior, including partner preferences and sexual conduct, are "organized" by estradiol. This study aimed at analyze these processes in the mandarin vole (*Microtus mandarinus*), a novel experimental mammal with strong monogamous pair bonds. Female pups were treated daily with an oil vehicle (FC) or β-Estradiol (E_2, FT) from prenatal day 14 to postnatal day 10. Male pups were treated daily with the oil vehicle only (MC). Partner preferences, sexual conduct and the expression of estrogen receptors α (ERα) and β (ERβ) were examined when animals were 3 months old. FT and MC groups showed female-directed partner preferences and masculinized behavior. ERα-immunoreactive neurons (ERα-IRs) in the bed nucleus of stria terminalis (BNST) and medial amygdaloid nucleus (MeA) was greater in FT females than MC males, and there was no significant difference in the number of ERα-IRs between FT and FC females. No difference was found for ERα-IRs in the preoptic area (mPOA) or ventromedial nucleus of the hypothalamus (VMH) of FT females or MC males, and they were significantly fewer than in FC females. ERβ-immunoreactive neurons (ERβ-IRs) in these four brain regions did not alter the ERβ/ERα ratio in different brain regions during perintal developments. However, the number of ERβ-IRs in FT females and MC males were greater than in FC females. We propose that estradiol treatment during perinatal development is responsible for adult partner preferences and mating behavior.

Key words: Estrogen receptor α (ERα), Estrogen receptor β (ERβ), Partner preference, Defeminization, Masculinization.

BACKGROUND

Estrogen exerts potent and wide-ranging effects on the developing brain (McCarthy 2008). It has been well recognized that the neural mechanisms that control mate preference and sexual conduct are sexually differentiated perinatally by sex steroid hormones (Henley et al. 2010). In males, gonadal steroid action is required early in development for adult steroids to effectively induce male sexual conduct. As for females, the lack of early exposure to high levels of gonadal steroids is of great importance for partner preference and sexual conduct (McCarthy 2008). Suppose that a developing female rat is inadvertently exposed to gonadal steroid hormones or mimetic agents, as an adult; she will lack sexual receptivity and display female-directed

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partner preferences (McCarthy 2008). As for female mice, exposure to prenatal estrogens defeminizes them, leading to decreased lordosis behavior and no clear mate preferences in adulthood (McCarthy 2008). Conversely, in species with monogamous mating systems, for instance prairie voles, this developing system seems to be insensitive to estrogens or exogenous androgens. Such insensitivity is atypical for a sexually dimorphic neural system in a rodent, and it may reflect the unusual effects of hormones on sexual differentiation of some behaviors (Lonstein et al. 2005). However, a recent study in prairie voles found that sex-specific colonization of the hippocampus and amygdala by microglia change when the vole is exposed to the synthetic estrogen ethinyl estradiol (EE) or Bisphenol A (BPA) during development (Rebuli et al. 2016). In other species with monogamous mating systems, such as pine voles, exposure to estrogenic diethylstilbestrol (DES) prenatally and neonatally changes female adult neural phenotypes and behavior related to monogamy (Engell et al. 2006). Taken together, ambiguity about the role of perinatal estradiol signaling in the development of mate preferences in female rodents persists (Henley et al. 2010).

The direction of estrogen effects on behavior can be modulated according to the levels of estrogen receptor subtypes (ERs). At least two ERs are as follows: estrogen receptor β (ERβ) and estrogen receptor α (ERα) (He et al. 2012). Early estrogen treatment affects ER levels. Estradiol benzoate treatment of newborn C57BL/6J female mice completely masculinized cell number of ERs in the bed nucleus of the stria terminalis (BNST) during adulthood (Hisasue et al. 2010). Blocking estrogen action during development through ER knock outs results in a decrease in male rats' preferences for females (Wersinger and Rissman 2000; Henley et al. 2009). Male mice in which the ERα gene has been knocked out (ERαKO) have no preference for an estrous female over a male; estrous females, however, are strongly preferred by wild-type males (Wersinger and Rissman 2000). This defeminization of sexual conduct is possibly mediated by estrogen signaling through ERβ. The presence of castrated ERβ null males primed with estrogen and progesterone enhanced typical receptive female behavior when compared with wild-type males, indicating that ERβ signaling is essential for the defeminization of male behavior (Scordalakes et al. 2002). A recent study revealed that neural ERβ fails to play a crucial role in the organization and activation of the neural circuitry underlying male mice sexual conduct (Naulé et al. 2016) and whether neural ERβ plays a role in other animals needs further work.

No much knowledge is available on the brain systems mediating mammalian partner preferences. At least for non-primate species, the consensus is that chemical signals, transduced by the main olfactory and vomeronasal systems, are involved in displaying partner preferences, with one or the other playing a more essential role in a species-specific fashion (Bakker et al. 2003; Hamson et al. 2009; Henley et al. 2011). The medial preoptic area (mPOA), receiving main olfactory and vomeronasal information from the amygdala and BNST, has been connected to the display of male sexual conduct in an impressive number of vertebrate species (Henley et al. 2011). Estrogen receptors are expressed in these brain regions (Rolls 2004). Information on chemical signals reaches the hypothalamus (VMH) through the amygdala, which is involved in displaying proceptive and receptive behaviors (de Vries and Sodersten 2009), and lesions of the medial amygdala (MeA) diminish proceptivity in female rats (Gerardin et al. 2006). Regarding partner preferences, damage to VMH in female rats (de Vries and Sodersten 2009) and ferrets (Gerall 1967) after adult ovariectomy and estradiol treatment cut down on the tendency of females to approach males or their odors. Likewise, a female's preference for an intact male over a castrated male is enormously reduced by ERα knockdown in VMH of rats after adult ovariectomy and ovarian hormone replacement (Hamson et al. 2009). A circuit from the main and accessory olfactory bulbs to VMH may be introduced in the sensory processing and integration of signals from conspecifics guiding female partner preferences (Henley et al. 2011). However, the difference in ERα and ERβ distribution in these brain regions in females with different partner preferences and sexual conduct has not been reported.

The majority of studies investigating the perinatal developmental effects on adult partner preferences has been done on rats and mice (Henley et al. 2011). Here, we instead studied mandarin voles (*Microtus mandarinus*) because rats and mice are socially polygamous and the mandarin vole is socially monogamous (Tai et al. 2001; Tai and Wang 2001). Male and female mandarin voles display a high level of social behavior and form selective partner preferences (Carter et al. 1995; Guo et al. 2011) Valuable insight into the neurobiological mechanisms that meidiate partner preferences can be provided by the research in this socially monogamous rodent (Cushing et al. 2004). First, this study aimed to determine whether adult female mandarin voles receiving exogenous estradiol during development show female-directed partner preferences and defeminization. Second, we compared the distribution of ERα and ERβ in the mPOA, BNST, MeA and VMH in female mandarin voles with female-directed partner preferences and males.

MATERIALS AND METHODS

Animals

Healthy adult females and males (n = 30, 30–36 g, 90 days old) were obtained from an outbred colony and reared at the College of Life Sciences, Shaanxi Normal University, Xi'an, China. This colony of mandarin voles was established in 1997 with wild-captured animals from Lingbao City, Henan, China (He et al. 2008). Animals were individually housed in clear plastic cages (30 × 20 × 15 cm) and maintained on a 14:10 h light: dark cycle at 24–26°C. Hardwood shavings and cotton were provided as substrate and bedding. Rabbit chow (Laboratory Animals Center, Xi'an Medical University, Xi'an, China), carrot and malt were provided *ad libitum*. All methods were approved by the Institutional Animal Care and Use Committee of Shaanxi Normal University (He et al. 2015). Each female was paired with an adult male with bilateral or unilateral descended testes (n = 30, total = 30 pairs) until two ejaculations were observed (day 0 of pregnancy) (Ward et al. 2002; He 2014).

Treatment

Pregnant dams were given either subcutaneous (S.C.) injection of β-Estradiol sesame oil mixtures (E 2758-250MG, Sigma, 5 mg/kg) or a single sesame oil 100 μl at 8:00–8:20 each morning from day 14 of pregnancy until postnatal day 0. We chose to inject this concentration of estrogen for two reasons. First, in previous studies, rats were injected subcutaneously with 5 mg/kg estradiol benzoate to up-regulate the distribution of androgen receptors, thereby affecting the animal's masculinization behavior (Lynch and Story 2000; Pereira et al. 2003); second, we have found through experiments that 5 mg/kg injection of β-Estradiol sesame oil mixtures is clearly tied to male masculinity (Unpublished). The gestation period of voles is 21 days. We provided voles with two different treatments at day 14 of their pregnancy because prenatal brain development (from day 14 of pregnancy, including day 14 until postnatal day 0) is of vital importance in rodents and we considered pregnancy time was determined after two ejaculations (Ward et al. 2002). For some female voles, there was still no pregnancy after two ejaculations. As a result, we treated many pregnant dams with two different treatments for different periods. For instance, pregnant voles were given either β-Estradiol sesame oil mixtures or sesame oil daily starting at day 0 before giving birth. However, the number of pregnant voles that were given these treatments on day 14 before birth was the largest in all treated pregnant voles, and the number was statistically important. Hence, only offspring from pregnant voles that accurately were given these treatments from day 14 to day 21 were used (He 2014). On postnatal day 0, female offspring continued to receive either an S.C. injection of β-Estradiol sesame oil mixtures or a single sesame oil 50 μl until postnatal day 10. The reason these animals were abandoned was that gonadal hormones have a developmental role in organizing nervous system that regulates sexually dimorphic behavior. The perinatal period was the neonatal critical period for development in masculinization or feminization of brain structure and function begins before birth and ends by postnatal day 10 (PN10) (Bonthuis et al. 2010).

Experimental females—Each female offspring received the same material given to her mother and was injected for 17 consecutive days (from prenatal day 14 to postnatal day 10). They were kept with their mothers until postnatal day 21, when weaning occurs. Then, female offspring were housed in a cage with 2–3 other females in the same treatment. Female offspring were left undisturbed until the onset of behavioral testing. At the age of approximately 3 months (weight 30–36 g), partner preference and sexual conduct were tested. Overall the study design included three groups of animals: a female control group (FC, n = 10) receiving injections of sesame oil for 17 consecutive days; a female treatment group 1 (FT, n = 10) receiving injections of β-Estradiol sesame oil mixture for 17 consecutive days; and a male control group (MC, n = 10) receiving injections of sesame oil for 17 consecutive days.

Stimulus Males

Sexually experienced gonadally intact adult male mandarin voles at least 90 days old with bilateral or unilateral descended testis were used as stimulus animals in behavioral tests.

Stimulus Females

Sexually experienced gonadally intact adult female mandarin voles at least 90 days old were used as stimulus animals in behavioral tests. The mandarin vole is socially monogamous, and it was difficult for the stimulus female to enter the ovulation period without a familiar male spouse; therefore, it was necessary to bring them into estrous with exogenous hormone injections. So prior to testing, female stimulus animals were brought into estrus with estradiol benzoate (0.00075 mg/g, 24 h before testing) and progesterone (0.015 mg/g, 4–6 h before testing), and the estrus state was monitored using vaginal smears, stained with

thionin and examined microscopically (He et al. 2013). Vaginal smears were rated as estrous if most of the cells were non-nucleated cornified cells with only a small number of epithelial cells. Only females in estrous were used as stimulus females (He et al. 2008; Meek et al. 2006). Stimulus males and females were often caged in a different animal housing unit, so the research subjects were never exposed to any male-derived or female-derived odor other than during the test. For each test, cages were taken randomly out of the housing unit to avoid the possibility that the same animals were always tested first or last (Bakker et al. 2002).

Behavioral tests

Partner Preference

Tests for partner preference (30-min duration) were conducted in a Y-shaped test apparatus consisting of three polycarbonate cages (20 × 25 × 45 cm). Two of the cages (stimulus) were placed in parallel with a third cage (neutral) attached separately to each stimulus cage by a plastic tube (15 cm in length and 7.5 cm in diameter) (Jia et al. 2008). The two parallel chambers housed the stimulus voles.

Partner Preference 1: The stimulus, including one intact male and one estrous female (see below), were anesthetized with sodium pentobarbital (40 mg/kg) prior to being placed in the parallel chambers.

Partner Preference 2: One intact male and one sexually receptive female stimulus animal (see below) were tethered individually to a bar at the front end of each parallel chamber using a 15 cm wire fitted with a swivel to limit the movement of the stimulus animal to its own chamber. Stimulus animals were adapted to the tether prior to testing (Henley et al. 2009 2010).

In these two experiments, the experimental female was able to move freely among the three chambers and make physical contact with stimulus animals. Experimental animals were adapted to the apparatus twice for 10 min each prior to testing (Henley et al. 2009 2010). In behavioral test 1, total partner preference included the duration of affiliation and sniffing the anesthetized animal (anogenital region, face and flank) in each chamber and duration spent in their own chamber. In behavioral test 2, total partner preference included the duration of affiliation, sniffing (anogenital region, face and flank), mount intromissions, ejaculations, and lordosis behavior shown by the experimental female towards stimulus animals and the duration they remained in their own chamber. Mounts, intromissions, ejaculations, and lordosis between the experimental and stimulus animals were quantified (Henley et al. 2009). All behavioral testing took place under dim red-light illumination in the middle part of the dark phase of the light-dark cycle. Behaviors were recorded for 30 min using a digital video camera and scored later by an experimentally blind rater (Jia et al. 2008). Data collected from all behavioral tests were analyzed by using Observer 5.0 (Noldus), a behavioral data acquisition program. Preference was scored by subtracting the amount of time spent in the stimulus male (anesthetized or awake states) chamber from the time spent in the stimulus female chamber. A positive preference score means more time spent with the stimulus female, whereas a negative preference score represents more time spent with the stimulus male. Before the initial partner preference test, experimental females were sexually naïve (Henley et al. 2010). The entire Y maze was washed with soap and water and wiped with 70% ethanol between test sessions for each subject (Kelliher and Baum 2001).

Female Sexual conduct

All experimental female voles, including FC and FT females, were brought into estrus with estradiol benzoate (EB, 0.75 lg/g, 24 h before testing) and progesterone (0.015 mg/g, 4–6 h before testing) (Swaab et al. 1995; He et al. 2012). Adult behavioral tests were run after EB and progesterone treatments in adulthood. This hormonal regime was used to test the females under one naturally occurring hormonal condition: estrogen plus progesterone, typical of late proestrus. Female voles mate during late proestrus, when ovarian hormones are present (Henley et al. 2009). The MC males had descended bilateral or unilateral testes. Tests for sexual conduct displayed by the experimental females were conducted in a Plexiglas observation chamber (20 × 25 × 45 cm).

During the test, the experimental female had unrestricted access to the stimulus male animal (see "Testing Schedule" section below for more details). Tests lasted 30 min (Henley et al. 2010). The behavioral test was conducted under dim red-light illumination in the middle of the dark phase of the light-dark cycle. A videotape was made for the test and the frequency of male mounts, intromissions and ejaculations were scored, as was the latency. The latency of experimental female to approach stimulus male (Henley et al. 2009), and sexual receptivity were also recorded. Female sexual receptivity was recorded by calculating the lordosis quotient (multiplied by 100) as the number of times a female exhibited lordosis divided by the number of mounts (Henley et al. 2010). Finally, proceptive behaviors, which include hopping and darting, ear wiggling, and approaching the male, were scored in the test (Henley et al. 2009).

Male-like sexual conduct

Tests for male-like sexual conduct displayed by experimental females were conducted in a Plexiglas observation chamber (20 × 25 × 45 cm) (Henley et al. 2009). In the test, the experimental female was given unrestricted access to a stimulus female. The tests took 30 mins. Behavioral testing took place under dim red-light illumination in the middle of the dark phase of the light-dark cycle. Video recordings of these tests were analyzed to determine the frequency of mounts, intromissions, and ejaculatory patterns shown by the experimental females and the latency showing these behaviors (Henley et al. 2009).

Testing Schedule

Experimental animals were tested twice per week for 6 weeks (Fig. 1). During the first test each week, females were tested with only an anesthetized estrous stimulus female and anesthetized sexually active stimulus male. For the second test, females were tested with only an awake estrous stimulus female and an awake sexually active stimulus male. The initial partner preference of female was tested in Week 1 (Fig. 1A). Each experimental female was given sexual and social experience with both male and female stimulus animals during Weeks 2 and 3, but data were not kept. Under these experience conditions, experimental females were partnered with stimulus animals for 30 mins, during which time sexual conduct could occur. During Week 4, half of the experimental females were tested for sexual conduct with a male and the other half with a female (Fig. 1B). The sex of the stimulus animals was reversed for Week 5 (Fig. 1C). Sexual conduct during Weeks 4 and 5 was recorded and scored. During Week 6, the female's final partner preference was assessed (Fig. 1D) (Henley et al. 2009).

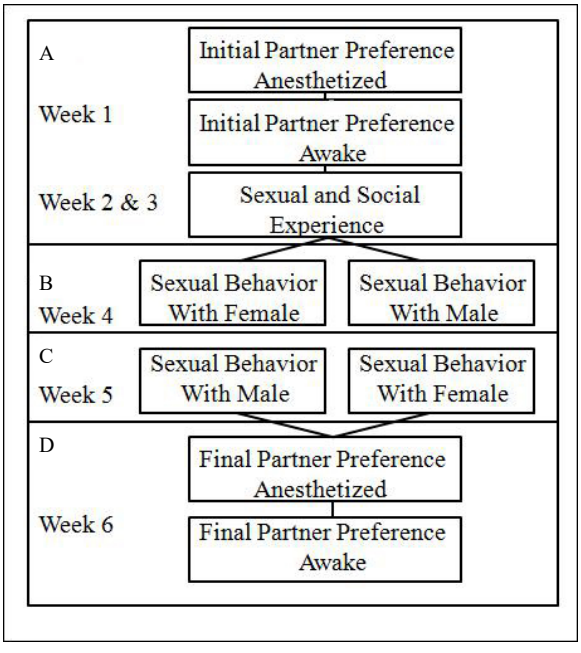

Fig. 1. Behavioral testing schedule for experimental females receiving perinatal injections of sesame oil (FC, $n = 10$) or β-estradiol sesame oil mixtures (FT, $n = 10$), and experimental males receiving perinatal injections of sesame oil (MC, $n = 10$). Data were not collected during weeks 2 and 3 during which the animals received sexual/social experience.

Enzyme-linked immunosorbent assay of serum E_2 in adult offspring

To avoid a change in hormonal data following any acute effects of these tests, blood samples were collected from the retro-orbital sinus between 08:00 and 10:00 two days after the female's final partner preference test (He 2014). No female was in estrus at the time of sacrifice. Most rodents like mandarin voles have a vaginal closure membrane which is perforated only at estrus and parturition (Kaiser et al. 2003). Thus, in mandarin voles, the condition of the vaginal membrane can be used as an external indication of estrus. Serum samples were separated from blood by centrifugation (3,000 rpm, 10 min) at room temperature and stored at -80°C before performing the assay (He et al. 2015). E_2 concentration in the serum was measured by using an enzyme-linked immunosorbent assay (ELISA, CEA461Ge, Cloud-Clone, USA). Serum samples were diluted 1:10 to measure E_2 (He and Tai 2009). First, the sample prepared and the standard were placed into the dish respectively and incubated for 30 mins at 37°C. Second, the dish was washed with washing solution for four times, and horseradish peroxidase (HRP)-blending agent was added and incubated for 30 mins at 37°C. Lastly, the dish was immersed in color developing Agent A and B after the additional dish was washed four times. After 15 mins incubation at 37°C, the reaction stopped by using stop solution. The optical density was measured at 450 nm by using a microplate reader (Bio-Tek, Winooski, USA) and the blank was set as zero. Variation between duplicate values was less than 5% (He et al. 2018).

ERα and ERβ immunohistochemistry

Brains were collected at the same time as blood; ERα and ERβ expressions were tested 2 days after behavioral test (He et al. 2013). Voles were deeply anesthetized and perfused with 0.1 M phosphate-buffered solution (PBS, pH 7.4) and 4%

paraformaldehyde in 0.1 M PBS. The brain was taken away within 3 mins and placed in 4% paraformaldehyde overnight. Before dissection, brains were put into 30% sucrose until saturated. Coronal sections (40 μm) were cut on a cryostat, and consecutive sections were collected in two vials containing 0.01 M PBS, to enable up to two different immunohistochemical staining assays (He et al. 2015). The antibody used for ERα (sc-542; Santa Cruz, CA, USA) and ERβ (Sc-8974, Santa Cruz, CA, USA) was an affinity purified rabbit polyclonal antibody of mouse origin raised against peptide mapping at the C-terminus of ERα and ERβ (He et al. 2012).

Floating sections were processed using primary antibody and streptavidin and peroxidase methods (Bioss Company, Beijing, China). Each vial of brain was incubated for 7 mins with 3% H_2O_2, the washed for 3 × 10 mins with 0.01 M PBS. Sections were pre-incubated for 90 min with normal goat serum (SP-0023) and incubated at 4℃ overnight with primary antibody solution (ERα antibody, 1:100; ERβ antibody, 1:100) diluted by antibody diluent (0.01 M PBS containing 20% bovine serum albumin and 1.7% Triton-X-100). The following day, sections were washed for 4 × 5 mins with 0.01 M PBS and incubated for 60 min in a 37℃ water bath with biotinylated goat anti-rabbit antibody (SP-0023), followed by 4 × 5 mins washing with 0.01 M PBS. After 60 min of incubation with streptavidin/horseradish peroxidase (S-A/HRP) and four washes for 10 mins each with 0.01 M PBS, sections were stained with 3,30-diaminobenzidine tetrahydrochloride (DAB) to visualize immunoreactivity. (He et al. 2013 2018)

Slides were randomized and coded for microscopic analysis so that counters were blinded to experimental treatment. The number of cells indicating immunoreactivity was quantified by eye per standard area (200 × 200 μm) using grid sampling. We counted the number of ERα-immunoreactive neurons (ERα-IRs) and ERβ- immunoreactive neurons (ERβ-IRs) in BNST, mPOA, and MeA in 40,000 μm^2. Different brain areas were decided according to Nissl-stained brain sections from mandarin voles and a stereotaxic atlas of the rat brain (Pellegrino et al. 1979; He et al. 2015).

For each brain nucleus, three typical sections from anterior to posterior and anatomically matched between subjects were selected and counted to minimize variability. Individual mean values for each animal were obtained by counting positive neurons bilaterally in three sections from each nucleus. Counts were separately performed for each hemisphere, and results were averaged between hemispheres. The left hemisphere was decided from the right hemisphere in accordance with morphological characteristics of the brain surface: within 3 min of removing the brain, we cut off a small part of the cortex in the left hemisphere and the right hemisphere as a template to discern the left hemisphere from the right. Sections were chosen based on the reference atlas plate instead of the level or intensity of ERα-IRs and ERβ-IRs labeling. All immunohistochemistry procedures included negative controls (the primary antibody was not added). A trained experimental rater blinded to experimental treatment counted positive neurons for all subjects. Selected sections were photographed with a Nikon camera (Tokyo, Japan) attached to a Nikon microscope (He et al. 2015).

Statistical analysis

For behavioral measures, data during the partner preference tests were analyzed using a 2 × 2 (perinatal treatment × initial or final test) ANOVA with repeated measurements of the second factor. The data for the behavioral measurements during the tests on sexual conduct, the expression of ERα, ERβ and serum E_2 levels were analyzed using an independent samples *t*-test (Henley et al. 2010). If a significant difference existed in the data, it was then followed by the post hoc Tukey method (He et al. 2018). Pearson's correlation coefficient was used to examine whether serum E_2 levels, ERα-IRs and ERβ-IRs correlated with preference scores and sexual conducts (Henley et al. 2010).

For some variables, the data failed to meet homogeneity of variance assumptions, even after transformation (*i.e.*, square root). Regarding these measurements, nonparametric statistics (Mann-Whitney *U*, Fisher's Exact Probability, and Wilcoxon Signed Ranks test) were used for analysis (Henley et al. 2010).

All data are presented as mean ± standard error (SEM) and significance was set at $P < 0.05$. Statistical analyses were made using SPSS10.0 (SPSS Inc., Chicago, USA) (He et al. 2012).

RESULTS

Behavioral results

Partner Preference (Partner Preference 1)

Perinatal treatment with estradiol altered the partner preference of females. A significant main effect of perinatal treatment with estradiol on partner preference was shown using 2 × 2 (perinatal treatment × initial or final test) ANOVA. Initial or final tests did not affect partner preference. The interaction between perinatal treatment and the initial or final test was significant for partner preference, and the effect of

perinatal treatment on partner preference was larger than the initial or final test. According to post hoc tests, FT females and MC males showed a higher preference score than FC females. The preference score was calculated as time spent with a stimulus female minus time spent with the stimulus male ($P < 0.05$, Fig. 2A). The FT females and MC males showed a higher total partner preference for stimulus anesthetized estrous females than FC females did ($P < 0.05$, Fig. 2B). FT females and MC males spent less time with the stimulus anesthetized intact male than did FC females ($P < 0.05$, Fig. 2C). Early estradiol treatments reduced preferences for the anesthetized intact male and increased preferences for the anesthetized estrous female.

Partner Preference (Partner Preference 2)

The above experiment further proved that perinatal treatment with estradiol changed partner preference of female experimental animals. A significant main effect of perinatal treatment with estradiol on partner preference was shown using 2 × 2 (perinatal treatment × initial or final test) ANOVA. The initial or final test did not affect partner preference. The interaction between perinatal treatment and the initial or final test was significant for partner preference, and the effect of perinatal treatment on partner preference was larger than the initial or final test. According to post hoc tests, FT females and MC males showed a higher preference score than FC females. Preference scores were calculated as time spent with the stimulus female minus time spent with the stimulus male ($P < 0.01$, Fig. 3A). FT females and MC males spent more time with the stimulus awake estrous female than FC females did ($P < 0.01$, Fig. 3B). FT females and MC males spent less time with the stimulus awake intact male than did FC females ($P < 0.01$, Fig. 3C). Early estradiol treatments reduced the preferences for the awake intact male and increased preference for the awake estrous female.

The proportion of females that displayed masculinized behaviors directed to the stimulus awake intact male during partner preference tests was affected by perinatal treatment with estradiol. The proportion of FT females receiving mounts, intromissions, or ejaculations from the stimulus awake intact male differed enormously among perinatal treatment groups (Table 1). Stimulus awake intact males indicated more sexual conduct toward FC females compared to FT ($P < 0.01$) and MC males ($P < 0.001$). MC males were aggressive and did not display sexual conduct towards the stimulus awake male (data were not collected) (Table 2).

The proportion of FT females ($P < 0.01$) and MC

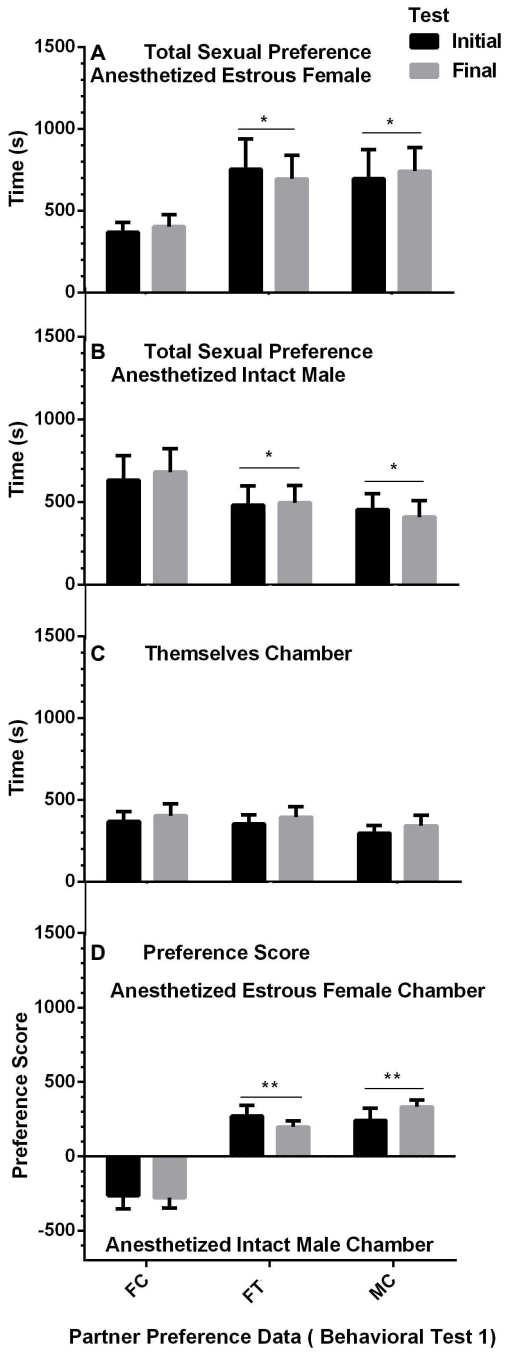

Fig. 2. Partner preference data (Behavioral Test 1). In Behavioral Test 1, experimental females were exposed to perinatal treatments and stimulus females or males were anesthetized. 2A) FT females and MC males spent more time with the stimulus anesthetized estrous female than the FC females did. 2B) FT females and MC males spent less time with the stimulus anesthetized intact male than did FC females. 2C) There was no difference in time spent in their own chamber for FC females, FT females, and MC males. 2D) FT females and MC males showed a higher preference score than that of FC females in the partner preference tests. Preference score is calculated as time spent with stimulus female minus the time spent with stimulus male. *: Significantly different from control group, $P < 0.05$; **: Significantly different from control group, $P < 0.01$.

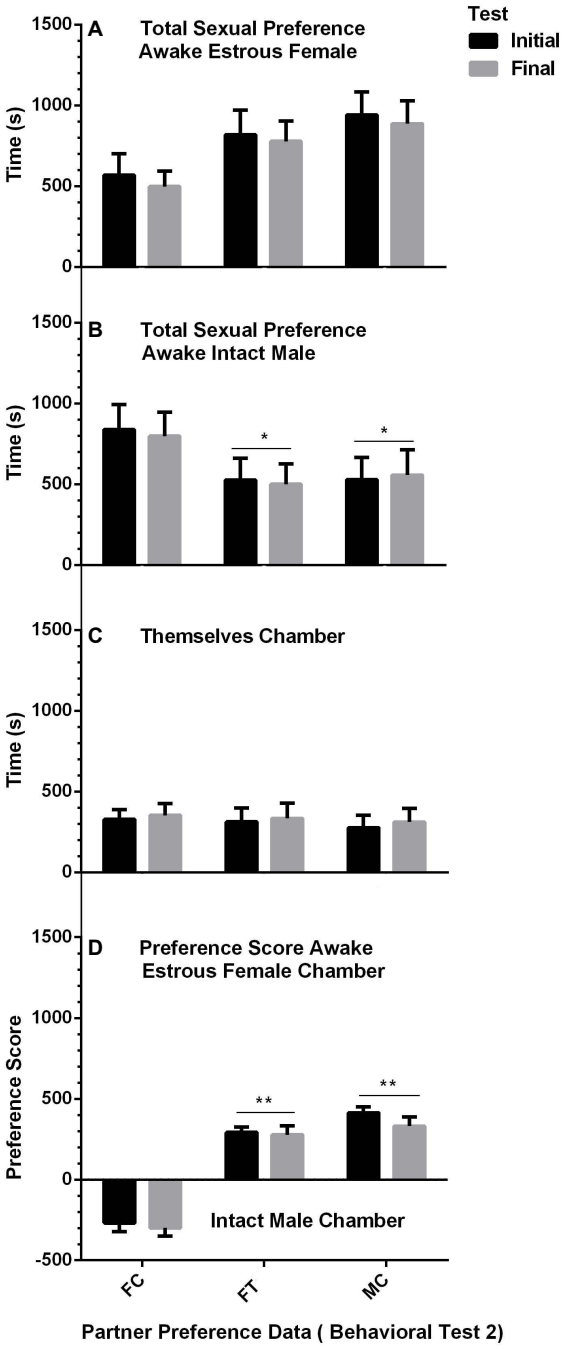

Fig. 3. Partner preference data (Behavioral Test 2). In Behavioral Test 2, experimental females were exposed to perinatal treatments and stimulus females or males were awake. 3A) There were no differences for the time spent in their own chamber for FC females, FT females and MC males. 3B) FT females and MC males showed a higher preference score than that of FC females in partner preference tests. Preference score is calculated as time spent with stimulus female minus the time spent with stimulus male. 3C) FT females and MC males spent more time with the stimulus awake estrous female than did FC females. 3D) FT females and MC males spent less time with the stimulus awake intact male than did FC females. *: Significantly different from control group, $P < 0.05$; **: Significantly different from control group, $P < 0.01$.

males ($P < 0.001$) that displayed masculinized behavior towards the stimulus awake estrous female during the partner preference test was higher than for FC females. Four (initial partner preference test) or five (final partner preference test) out of ten FT females revealed that, during the preference tests, full ejaculatory reflex pattern (push-pull action across female) was given, whereas none of the FC females displayed full ejaculatory reflex. FC females did not display sexual conduct towards stimulus awake estrus females (Table 3).

Female sexual conduct (feminized behaviors)

Nonparametric statistics were utilized to analyze the remaining measures to test female sexual conduct. A Mann-Whitney *U* test was utilized to compare behaviors of stimulus males on tests with FT females, MC males, and FC females treated as adults.

Perinatal treatment with estradiol in experimental females affected male mount, intromission, and ejaculation frequencies. Stimulus males showed fewer mounts, intromissions, and ejaculations in tests comparing FT and FC females ($P < 0.001$). Stimulus males did not display sexual conduct with MC males ($P < 0.001$) and did display more aggression than the other two groups (data were not collected) (Fig. 4). The proportion of experimental females receiving mounts, intromissions, and ejaculations by the stimulus male was analyzed using Fisher's exact probability test (Table 4).

Too few FT ($n = 2$) females were given at least three mounts (females bestrided the back of the stimulus female) for meaningful statistical comparisons of lordosis quotients (LQ) of FT vs. FC females. FT females received three mounts, but revealed no lordosis responses. In contrast, all FC females receiving at least eight mounts indicated lordosis responses with average LQs of 80% ($n = 10$). Proceptive behaviors were analyzed using the Mann-Whitney *U* test, which revealed that FT ($P < 0.001$) females displayed fewer proceptive behaviors than FC females (Fig. 5). Separate statistical tests were utilized to compare the FT and FC females for each specific proceptive behavior.

Male-like sexual conduct (masculinized behaviors)

Nonparametric tests were utilized to analyze data from the male sexual conduct tests. Significant differences were found between FT and FC females for behavioral measures, except LQ (Fisher's exact probability tests). However, six out of ten FT females showed the mount pattern, five out of ten FT females showed the intromission pattern, and five out of ten FT

Table 1. Main and interaction F-statistic values for analyses

	Two-way ANOVA					
	Perinatal treatment		Initial or final test		Interaction between both factors	
	$F_{2,27}$	P	$F_{1,28}$	P	$F_{2,27}$	P
Total sexual preference the stimulus anesthetized intact male (Initial test)	4.842	0.019	2.543	0.077	4.183	0.039
Total sexual preference the stimulus anesthetized estrous female (Initial test)	5.422	0.006	2.078	0.083	4.220	0.038
Total sexual preference the stimulus awake intact male (Initial test)	5.836	0.004	2.624	0.074	4.457	0.033
Total sexual preference the stimulus awake estrous female (Initial test)	4.442	0.034	1.767	0.158	3.678	0.046
Total sexual preference the stimulus anesthetized intact male (Final test)	4.426	0.035	2.543	0.077	3.939	0.043
Total sexual preference the stimulus anesthetized estrous female (Final test)	4.778	0.027	2.078	0.083	4.241	0.037
Total sexual preference the stimulus awake intact male (Final test)	5.432	0.006	2.624	0.074	4.538	0.032
Total sexual preference the stimulus awake estrous female (Final test)	4.461	0.033	1.767	0.158	3.479	0.047

Table 2. Proportion of experimental females that received sexual behavior from the stimulus awake intact male during the partner preference (Behavioral Test 2)

	Initial Partner Preference Test				Final Partner Preference Test			
Behavior Group	Lordosis Quotients	Mount	Intromission	Ejaculation	Lordosis Quotients	Mount	Intromission	Ejaculation
FC	83	6/10	5/10	3/10	90	7/10	6/10	4/10
FT1**	33	2/10	1/10	0/10	24	2/10	2/10	0/10
MC***	0	0/10	0/10	0/10	0	0/10	0/10	0/10

FT females were fewer likely than FC females to receive sexual behavior from the stimulus male. MC males did not display sexual behavior with the stimulus awake male during behavioral test 2. **: Significantly different from the FC females, $P < 0.01$. ***: Significantly different from the FC females, $P < 0.001$.

Table 3. Proportion of experimental females that revealed sexual behavior from the stimulus awake estrus female during the partner preference (Behavioral Test 2)

	Initial Partner Preference Test				Final Partner Preference Test			
Behavior Group	Lordosis Quotients	Mount	Intromission	Ejaculation	Lordosis Quotients	Mount	Intromission	Ejaculation
FC	0	0/10	0/10	0/10	0	0/10	0/10	0/10
FT1**	0	5/10	4/10	4/10	0	6/10	5/10	5/10
MC***	0	7/10	6/10	5/10	0	6/10	7/10	6/10

FT females and MC males were more likely to display male-like sexual behavior than FC females. FC females did not display sexual behavior with the stimulus awake estrus female. **: Significantly different from control group, $P < 0.01$. ***: Significantly different from control group, $P < 0.001$.

females presented the full ejaculatory pattern during the sexual conduct tests; no FC females did. Four out of these five FT females were the same females that showed ejaculatory patterns during partner preference tests. MC males displayed more mounts, intromissions and ejaculations and no lordosis with the stimulus female (Table 4).

Hormone Assays

Early hormone treatments ($X \pm$ SEM ng/dl: E_2: FC: 12.5 + 2.7, FT: 11.6 + 2.9, MC: 9.7 + 1.8) did not affect the circulating levels of estradiol. To test the possibility that circulating estradiol was directly accountable for the behavioral changes observed, a correlation was made between serum hormone levels and preference score. No significant correlation between preference score and E_2 level ($r = 0.13$, $P = 0.45$) was found.

Changes in ERα immunoreactivity in the mPOA, BNST, MeA, and VMH for different sexual preferences

The numbers of ERα-IRs in the mPOA ($F_{(2,27)} = 19.550$, $P < 0.001$), BNST ($F_{(2,27)} = 21.509$, $P < 0.001$), MeA ($F_{(2,27)} = 4.604$, $P < 0.05$) and VMH ($F_{(2,27)} = 6.750$, $P < 0.01$) varied with the treatment groups.

Post hoc tests revealed no significant gap in the number of ERα-IRs in the mPOA and VMH between

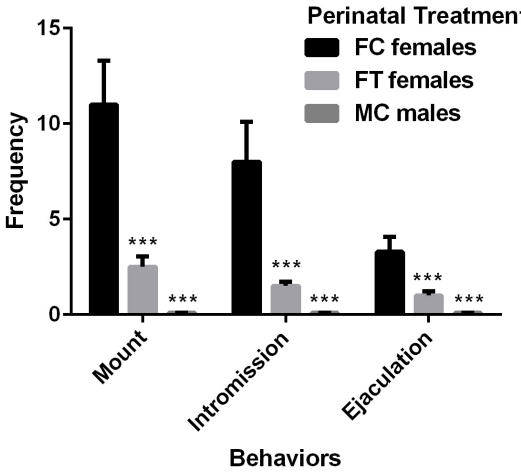

Fig. 4. Frequencies of mounts, intromissions, and ejaculations in females receiving sexual behavior tests. Males showed fewer behaviors when paired with FT females and MC males compared to FC females. **: Significantly different from control group, $P < 0.01$. ***: Significantly different from control group, $P < 0.001$. See text for details.

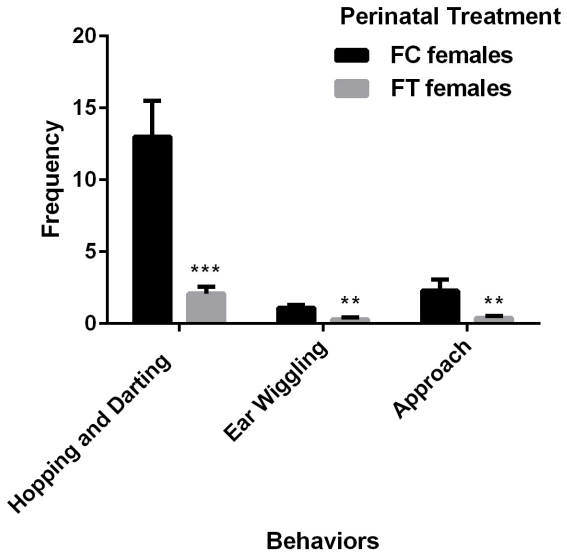

Fig. 5. Frequency of proceptive behaviors shown by experimental females during the female sexual behavior test. FT females exhibited fewer proceptive behaviors than FC females. ***: Significantly different from control group, $P < 0.001$. See text for details.

Table 4. Proportion of experimental females that received sexual behavior from the stimulus male or proportion of experimental females displayed male-like sexual behavior from the stimulus female during the sexual behavior tests (Behavioral Test 3 and 4)

	Sexual Behavior From The Stimulus Intact Male				Sexual Behavior From The Stimulus Intact Female			
Behavior Group	Lordosis Quotients	Mount	Intromission	Ejaculation	Lordosis Quotients	Mount	Intromission	Ejaculation
FC	80	8/10	7/10	5/10	19	0/10	0/10	0/10
FT1	20**	3/10**	2/10**	0/10***	21	6/10**	5/10***	5/10***
MC	0***	0/10***	0/10***	0/10***	0***	7/10***	6/10***	6/10***

In behavioral test 3, FT females receive fewer lordosis, mounts, intromissions and ejaculations from the stimulus male. MC males did not display sexual behavior. In behavioral test 4, FT females displayed more mounts, intromissions and ejaculations and less lordosis with the stimulus female. MC males displayed more mounts, intromissions and ejaculations and no lordosis with the stimulus female.

FT females and MC males ($P > 0.05$), and they were much fewer than FC females (mPOA: $P < 0.001$; VMH: $P < 0.01$). However, in FT and FC females, more ERα-IRs were found than in MC males in BNST and MeA (BNST: $P < 0.001$; MeA: $P < 0.05$), and no significant difference was found in the number of ERα-IRs in BNST and MeA between FT and FC females ($P > 0.05$) (Fig. 6).

Changes in ERβ immunoreactivity in the mPOA, BNST, MeA, and VMH for different sexual preferences

The number of ERβ-IRs was different among the mPOA ($F_{(2,27)} = 22.135$, $P < 0.001$), BNST ($F_{(2,27)} = 6.758$, $P < 0.01$), MeA ($F_{(2,27)} = 5.056$, $P < 0.01$), and VMH ($F_{(2,27)} = 4.079$, $P < 0.05$) in different treatment groups. Post hoc tests revealed that the number of ERβ-IRs in mPOA, BNST, MeA and VMH in FT females was quite similar to those in MC males ($P > 0.05$). However, the number of ERβ-IRs in FT females and MC males was greater than in FC females (mPOA: $P < 0.001$; BNST: $P < 0.001$; MeA: $P < 0.05$ and $P < 0.01$; VMH: $P < 0.05$) (Fig. 7).

Correlations between anesthetized animal preference scores and serum E_2 levels, as well as expression of ERα and ERβ in brain regions

According to the Pearson correlation analysis, preference scores for anesthetized animals (initial or final test) correlated positively to the levels of ERα and ERβ expression in mPOA, BNST, MeA, and VMH. Interestingly, no correlation between preference scores for anesthetized animals (initial or final test) and E_2 level was seen (Fig. 8).

Correlations between awake animal preference scores and serum E_2 levels, as well as expression of ERα and ERβ in brain regions

Pearson correlation analysis revealed that preference scores for awake animals (initial or final test) were positively correlated with expression of ERα and ERβ in the MeA, mPOA, BNST, and VMH. However, no significant correlation between preference scores for awake animals (initial or final test) and E_2 level was seen (Fig. 9).

Correlations were found between sexual conduct towards stimulus awake intact males and serum E_2 levels, as well as the expression of ERα and ERβ in brain regions, and between sexual conduct towards intact males and serum E_2 levels, and expression of ERα and ERβ in brain regions

Pearson correlation analysis indicated that levels of sexual conduct towards the stimulus awake intact male (initial or final test) during the partner preference and that towards the intact male correlated positively with levels of ERα and ERβ expression in the MeA, mPOA, BNST, and VMH. However, no significant correlation between sexual conduct towards the stimulus awake intact male (initial or final test) during the partner preference and sexual conduct with intact male and serum E_2 levels was seen (Fig. 10).

Correlations were found between sexual conducts with the stimulus awake estrous female during the partner preference and serum E_2 levels, as well as the expression of ERα and ERβ in brain regions, and between sexual conducts with estrus female and serum E_2 levels, and expression of ERα and ERβ in brain regions

Pearson correlation analysis indicated that levels of sexual conduct with the stimulus awake estrous female (initial or final test) during the partner preference and sexual conducts with estrous female correlated positively with levels of expression of ERα and ERβ in MeA, mPOA, BNST, and VMH. However, no significant correlation between sexual conduct with the stimulus awake estrous female (initial or final test) during the partner preference and sexual conducts with estrous female and serum E_2 levels was found (Fig. 11).

DISCUSSION

Estrogen treatment during development alters adult female sexual preferences

Overally, these experiments show that female mandarin voles exposed to exogenous estrogen during the early development presented alter partner preferences and increase masculinize behavior as adults. Compared to FC females, FT females and MC males showed increased preference for stimulus anesthetized or awake estrous females. Body odorants of anesthetized or awake voles (*e.g.*, urinary pheromones, extraorbital lacrimal gland secretions (Dibenedictis et al. 2014), and anal scent gland secretions) play an essential part in sex discrimination and attraction between males and females, which influences mate choice (Baum and Kelliher 2009). Body odorant processing is linked to sexual preference (Baum 2006), and estrogens during

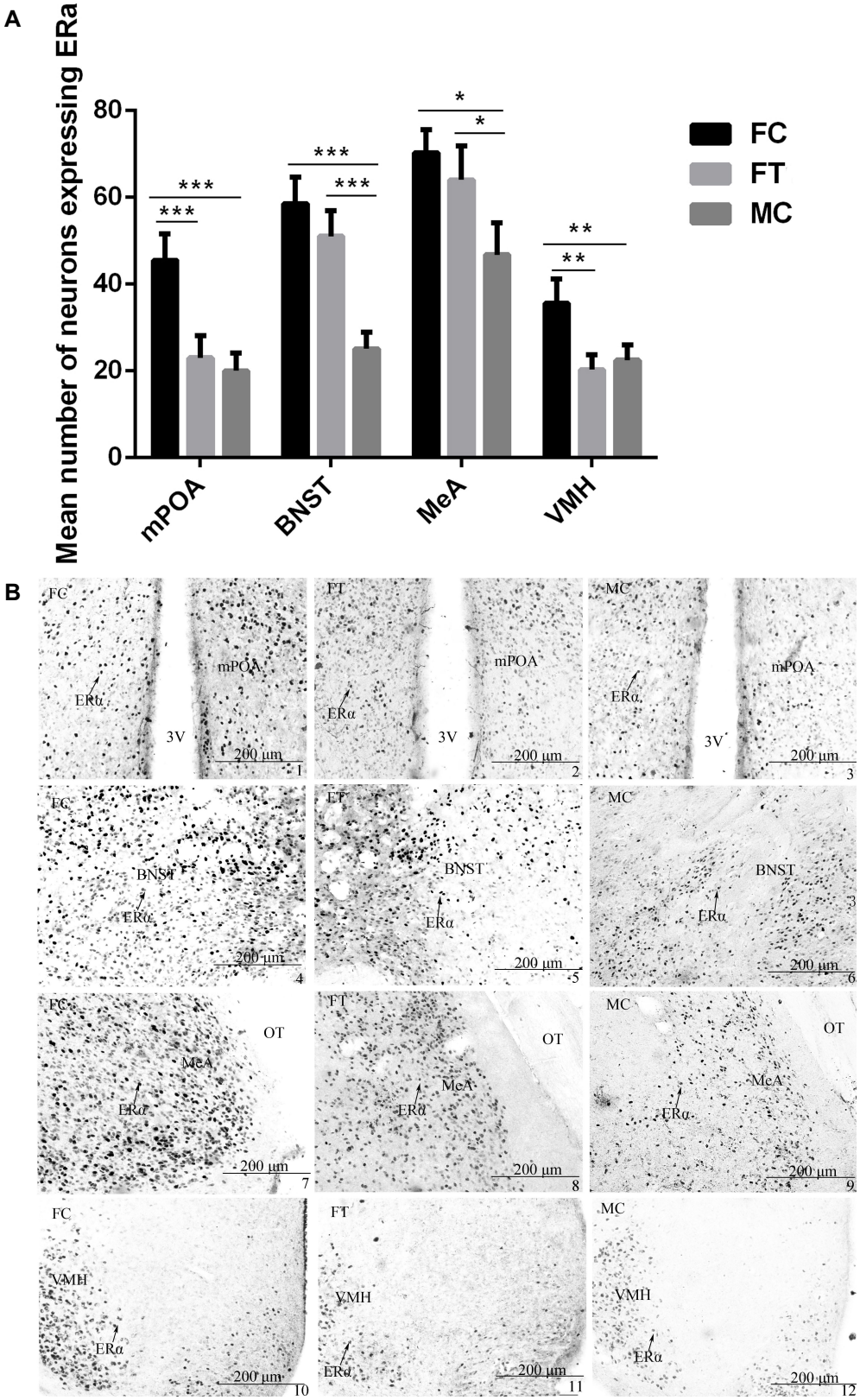

Fig. 6. Mean (± SE) number of ERα-IRs in FC, FT, and MC groups. mPOA: medial preoptic area. BNST: bed nucleus of the stria terminalis. MeA: medial amygdaloid nucleus. VMH: ventromedial nucleus of the hypothalamus. 3V: third ventricle. OT: optic tract. Scale bar = 200 μm. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

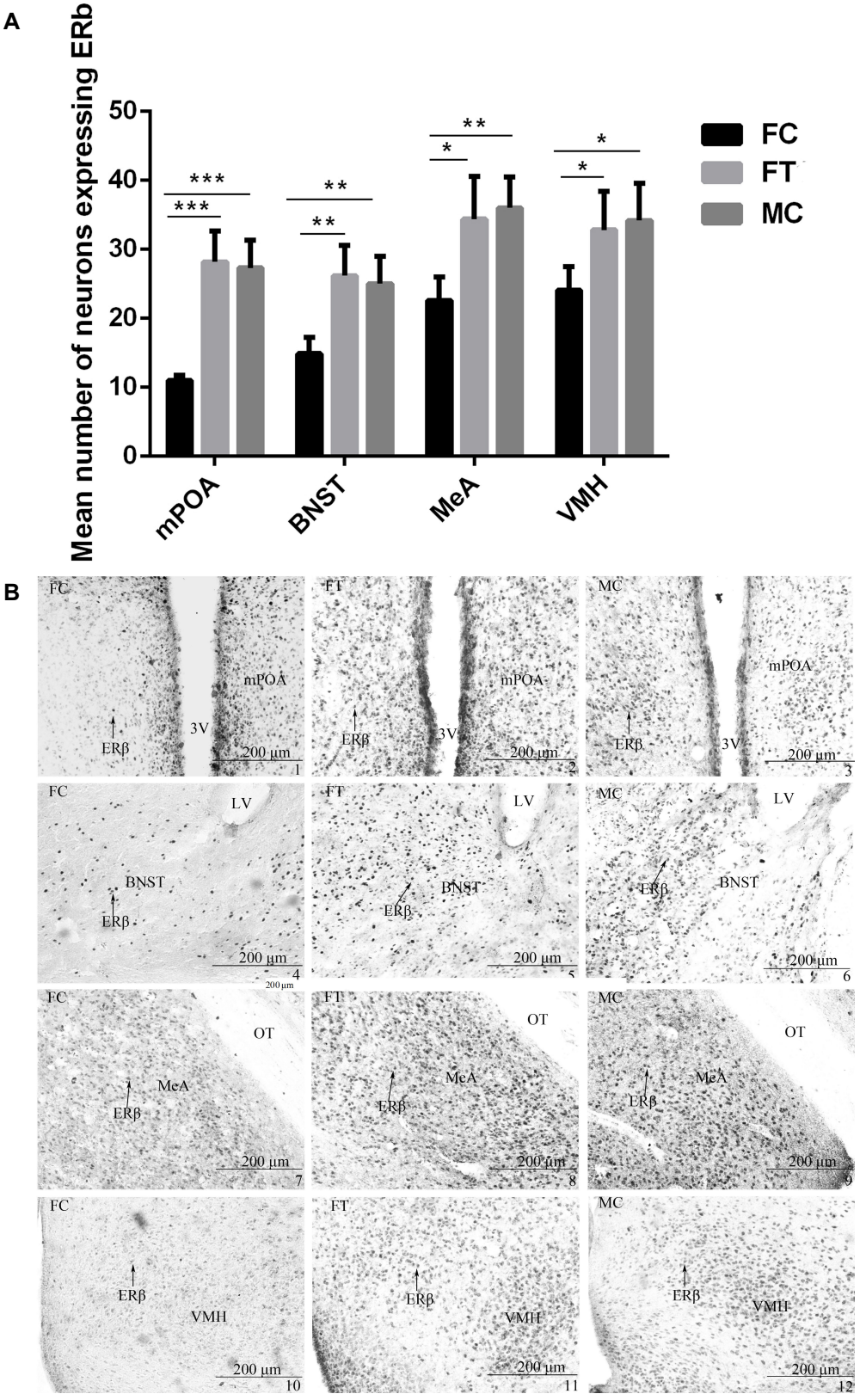

Fig. 7. Mean (± SE) number of ERβ-IRs in FC, FT, and MC groups. mPOA: medial preoptic area. BNST: bed nucleus of the stria terminalis. MeA: medial amygdaloid nucleus. VMH: ventromedial nucleus of the hypothalamus. 3V: third ventricle. LV: lateral ventricle. OT: optic tract. Scale bar = 200 μm. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

development are needed to organize the olfactory pathways involved in partner preferences (Bonthuis et al. 2010). We confirmed that perinatal estradiol exposure during development produced female-directed partner preferences in adult females. This result is consistent with a study in female rats showing that female-directed partner preference depends on early exposure to estrogens in female rats (Henley et al. 2009). Other studies showed that female rats that received exogenous estradiol during development spent more time with an estrous female and less time with a sexually active male than did control females (Henley et al. 2010). Early estrogen exposure mediating a preference for females is clear in the human literature. Diethylstilbestrol (DES) is a nonsteroidal synthetic estrogen and DES-exposed women have a higher incidence of bisexuality or homosexuality than non-exposed women (Ehrhardt et al. 1985; Henley et al. 2009). However, female mice with a deficiency in alpha-fetoprotein (AFP-KO) failed to completely resemble males in their mate preferences, which suggests that the male-typical pattern of mate preferences is not solely organized by prenatal estrogens (Brock and Bakker 2011). The early postnatal period is a time during which the process of sexual differentiation is particularly sensitive to endogenous and environmental challenges (Henley et al. 2010). Female

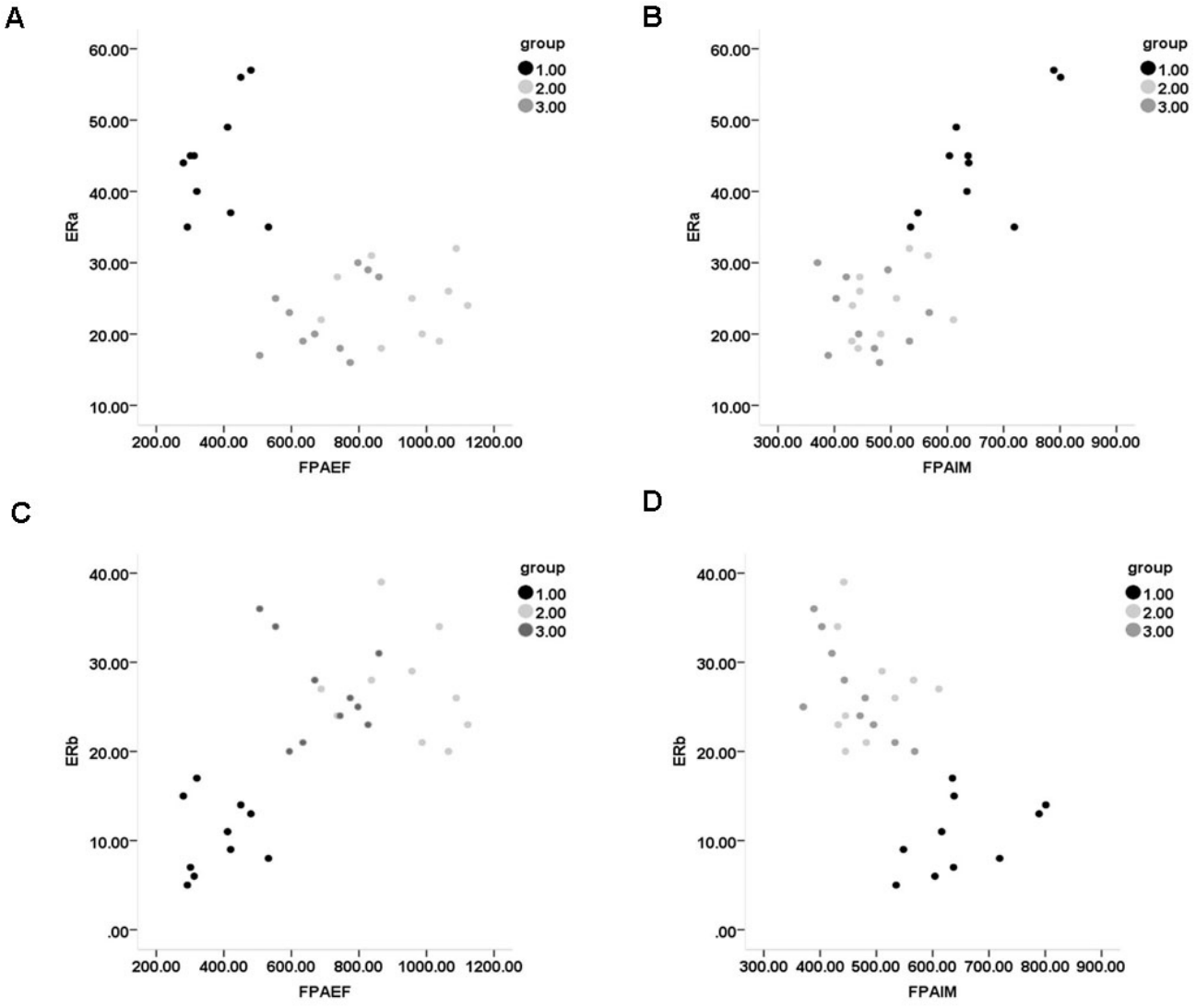

Fig. 8. Correlations behaviors between preferences scores of anesthetize animals and ERα, as well as ERβ in mPOA in FC, FT and MC groups. mPOA: medial preoptic area; ERa = estrogen receptor-α; ERb = estrogen receptor β; 1 = FC; 2 = FT; 3 = MC; FPAEF = final preference anesthetize estrous female; FPAIM = final preference anesthetize intact male; A: Correlations between ERa mPOA and FPAEF, r = -0.610, $P < 0.001$; B: Correlations between ERa mPOA and FPAIM, r = 0.790, $P < 0.001$; C: Correlations between ERb mPOA and FPAEF, r = 0.636, $P < 0.001$; D: Correlations between ERb mPOA and FPAIM, r = -0.678, $P < 0.001$.

mandarin voles may have similar social behaviors to female prairie voles and be susceptible to influence from gonadal hormones during development, including the prenatal and early postnatal period (Lonstein et al. 2005). Our experimental female voles received estrogen treatment from prenatal day 14 until postnatal day 10 (17 consecutive days), and we posit that the critical period for mandarin vole brain sexual differentiation may be prenatal and early postnatal days.

Perinatal estrogen treatment also affected the sexual conduct of female voles. FT reduced the time females spent with males during the sexual conduct test, and very few FT females indicated lordosis in response to male mounts. This effect on female receptivity is in agreement with other studies showing that neonatal estrogen treatment reduces the frequency of lordosis in females (Henley et al. 2009). Estradiol treatment disrupted normal female sexual conduct, receptivity, and proceptivity (Henley et al. 2009). The proportion of FT females and MC males that engaged in mounting behavior when paired with a female was higher than in the FC. The increase in mounts shown with a stimulus female or male suggests behavioral masculinization. Exposure to estrogen during development is responsible for both masculinization and defeminization of the brain and behavior (Henley et al. 2010).

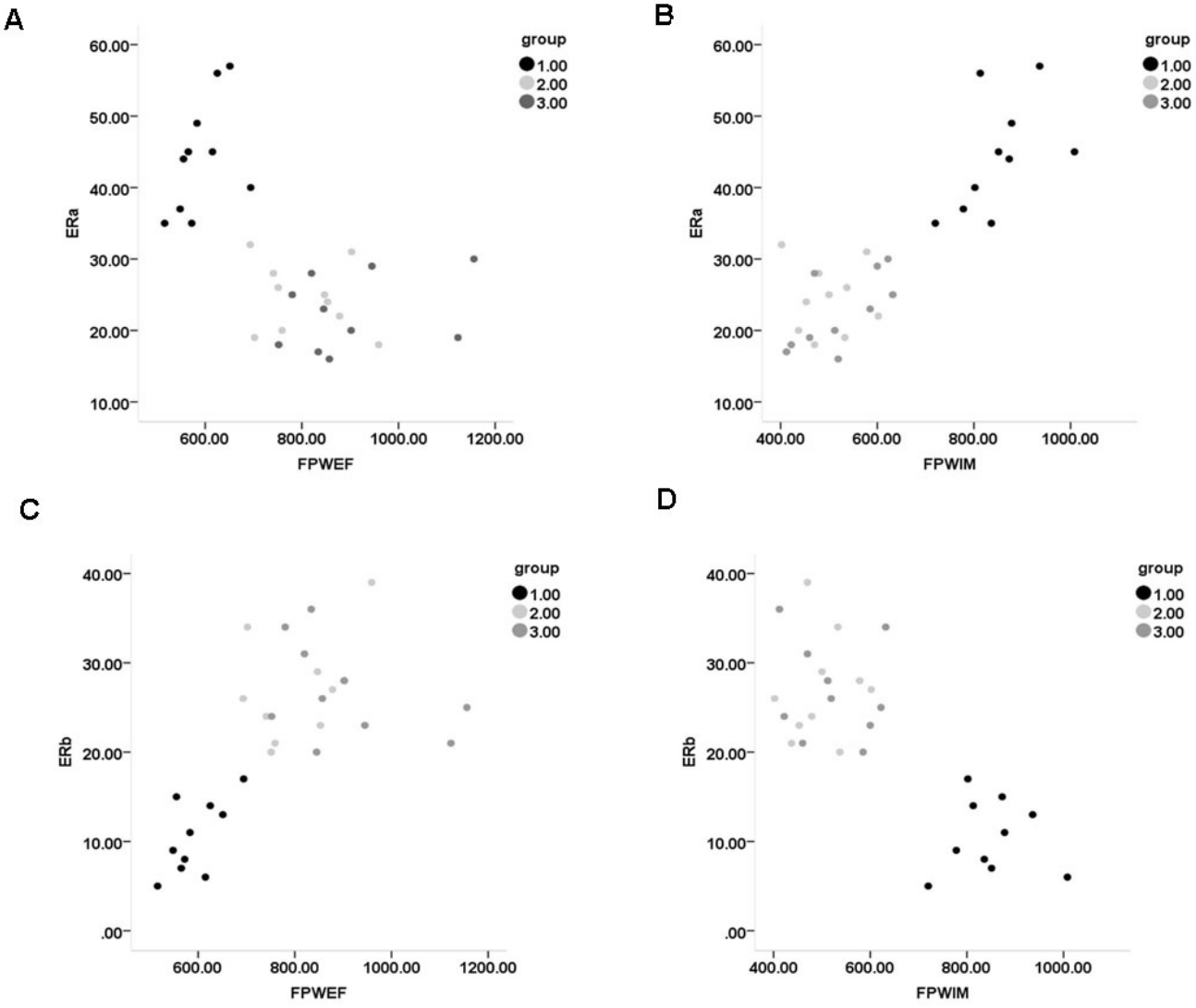

Fig. 9. Corrolations behaviors between preferences scores of awake animals and ERα, as well as ERβ in mPOA in FC, FT and MC groups. mPOA: medial preoptic area; ERa = estrogen receptor-α; ERb = estrogen receptor β; 1 = FC; 2 = FT; 3 = MC; FPWEF = final preference awake estrous female; FPWIM = final preference awake intact male; A: Corrolations between ERa mPOA and FPWEF, r = -0.625, $P < 0.001$; B: Corrolations between ERa mPOA and FPWIM, r = 0.864, $P < 0.001$; C: Corrolations between ERb mPOA and FPWEF, r = 0.659, $P < 0.001$; D: Corrolations between ERb mPOA and FPWIM, r = -0.744, $P < 0.001$.

In the partner preference tests and female sexual conduct test, FT females were less likely than FC females to receive mounts, intromissions, or ejaculations from the stimulus male. Such reduced interest by males for FT females could reflect multiple factors that are not mutually exclusive. FT females could be less proceptive; they could be less attractive to males, or they could actively avoid males, any of which would lead to decreased sexual interest and sexual conduct by the stimulus male. Proceptive behaviors were measured during the female sexual conduct test, and FT females engaged in less ear wiggling, hopping, darting, and approaches to the stimulus male compared to FC females. Attractivity was not measured in this study, but could also be an explanation for the low frequency of male behaviors directed toward FT females. Stimulus males may find FT females less attractive than FC females, leading to fewer sexual interactions. Finally, avoidance behaviors were not measured, but the lack of sexual interactions with the male may be attribute to FT females actively avoiding contact.

The present experiment also showed that control females displayed male-directed partner preferences and sexual conduct. This is consistent with the results of other studies in most animals showing a preference for a stimulus male over a female (Henley et al. 2011).

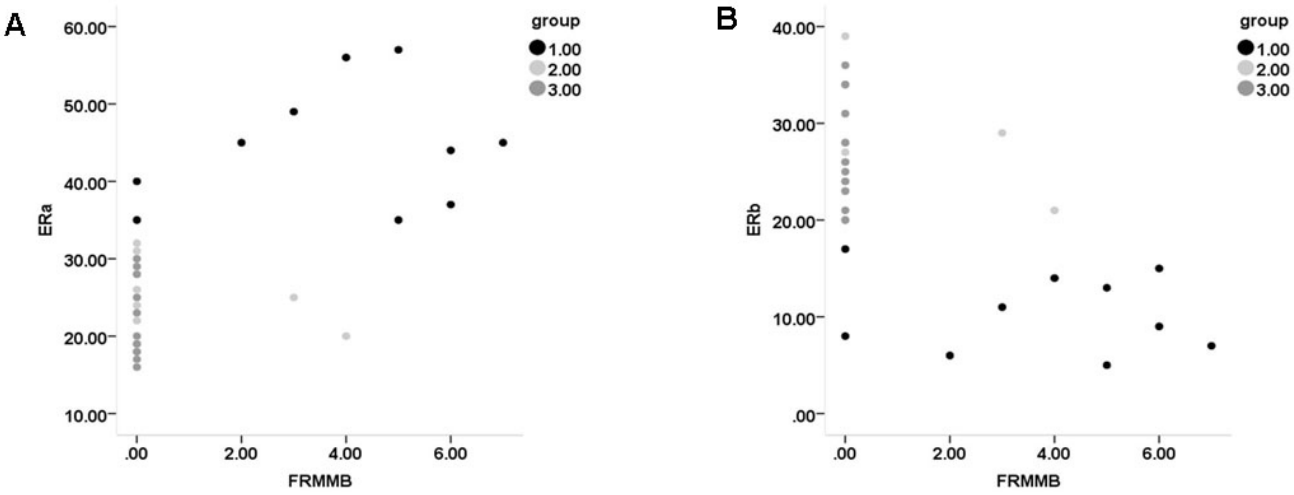

Fig. 10. Corrolations between received mount behavior with the stimulus awake intact male during partner preference and expression of ERα, as well as ERβ in mPOA in FC, FT and MC groups. mPOA: medial preoptic area; ERa = estrogen receptor-α; ERb = estrogen receptor β; 1 = FC; 2 = FT; 3 = MC; FRMMB = receiving mount with the stimulus awake intact male during final partner preference test; A: Corrolations between ERa mPOA and FRMMB, $r = 0.645$, $P < 0.001$; B: Corrolations between ERb mPOA and FRMMB, $r = -0.651$, $P < 0.001$.

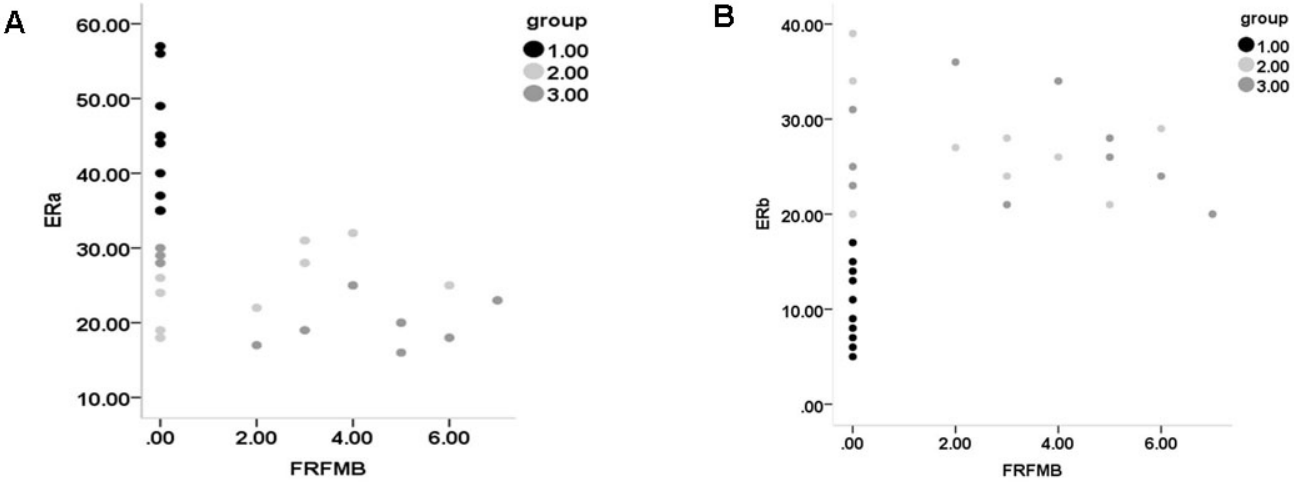

Fig. 11. Corrolations between revealing mount behavior with the stimulus estrous female during partner preference and expression of ERα, as well as ERβ in mPOA in FC, FT and MC groups. mPOA: medial preoptic area; ERa = estrogen receptor-α; ERb = estrogen receptor β; 1 = FC; 2 = FT; 3 = MC; FRFMB = revealing mount with the stimulus awake estrous female during final partner preference test; A: Corrolations between ERa mPOA and FRFMB, $r = -0.545$, $P = 0.002$; B: Corrolations between ERb mPOA and FRFMB, $r = 0.371$, $P = 0.044$.

In the above experiment, measures of behavioral experiments failed to alter largely from the first to second partner preference test. This is inconsistent with the report that, regardless of postnatal estradiol exposure during development in rats, a number of behavioral measures were significantly different between the first and second partner preference tests in all experiments (Henley et al. 2009 2010). Changes in partner preference test may attribute to the sexual experience received, or it could have been a result of experimental animals being more familiar with the testing apparatus and stimulus animals (Henley et al. 2010). In this study, partner preference may be a preferred indicator of motivation. These discrepancies could be species-dependent or result from other factors (Henley et al. 2010; Brock et al. 2015)

Effects of ERα in the mPOA, BNST, MeA, and VMH on partner preference and sexual conduct

The results support our previous findings that the mPOA, BNST, MeA, and VMH in FC females have a higher ERα density than MC males (He et al. 2016). In socially monogamous prairie (*Microtus ochrogaster*) and pine (*M. pinetorum*) voles, ERα expression in the MeA and BNST is sexually dimorphic (Cushing et al. 2004, Cushing and Wynne-Edwards 2006; Perry et al. 2016). The BNST and MeA regulate social behavior, including social preference, a critical aspect of pair-bond formation, affiliation, and aggression (Cushing et al. 2004). Therefore, low levels are 'necessary' for the expression of social behavior in males (Cushing et al. 2008; Perry et al. 2016). Sex differences (female > male) in ERα-IRs were observed not only during the prepubertal period in the BNST and the mPOA, but also in adulthood in these two brain regions (Nakata et al. 2016). MC males showed an increased partner preference for stimulus anesthetized or awake estrous females and is consistent with the report that ERα in the MeA of male prairie voles formed a partner preference for a novel female (Cushing et al. 2008). That FC females, which more ERα-IRs in the BNST and MeA than MC males is consistent with the report that the number of ERα-IRs was sexually dimorphic in the highly social monogamous pine vole, with females expressing more ERα-IRs than males in brain regions including the BNST and MeA (Cushing and Wynne-Edwards 2006). However, the sexually dimorphic distribution of ERα-IRs in the BNST and MeA and association with partner preferences and sexual conduct is unclear in pine voles.

Although FT2 females had altered partner preferences and increased masculine behavior as adults, there was a different distribution between FT females and MC males for ERα-IRs in the BNST and MeA. More ERα-IRs in the BNST and MeA were found in FT females than MC males. Our results are consistent with the expression pattern of ERα in female guinea pigs with behavioral masculinization. In male guinea pigs, fewer ERα-IRs were found than in masculinized female and control female guinea pigs (Kaiser et al. 2003). However, FT females had fewer ERα-IRs in the mPOA and VMH than FC females, and this expression pattern of ERα in FT females was similar to that of MC males. In ERα expression patterns throughout the hypothalamus, distribution differences could reveal differences in regional sensitivities to estrogen, and could thus indicate that estrogen, acting via ERα, affects these hypothalamic regions (the BNST, mPOA, MeA, and VMH) differently based on estradiol treatment during perinatal development (Brock et al. 2015). The male-typical patterns of ERα expression in mPOA and VMH are related to the behavioral and endocrine masculinization of early estradiol exposure females, since these brain areas are well known to play an essential part in controlling masculine behavior (Kaiser et al. 2003), sexual preference, and the regulation of gonadotropin releasing factors (Fernández-Guasti et al. 2000). Our results are consistent with several reports in the rat and ram that show estradiol exposure during the first few days of life reduces hypothalamic ERα expression in adults (Handa et al. 1996; Perkins et al. 1995). E_2 downregulates ERα at the level of gene expression (Simerly and Young 1991) and ERα-IRs and this could explain fewer ERα-IRs in FT2 females' mPOA and VMH. Further studies are required to determine whether increases in ERα are maintained or undergo additional modification during adolescence and adulthood (Kramer et al. 2007). These differences may be related to species and research factors.

Estrogen concentrations did not differ between the two categories of females. The above conclusion backs up the notion that in females local estrogen provision impacts brain function and behavior independent of ovarian steroids (Henley et al. 2010). Between serum estradiol levels and preference scores, no significant correlation was found, indicating that variations in the level of circulating hormone appear less useful in explaining adult behavior (Henley et al. 2010). Neonatal hormone exposure may create lasting differences in ERα expression (Kurian et al. 2010).

Effects of ERβ in the mPOA, BNST, MeA, and VMH on sexual partner preference and sexual conduct

Present results support our previous findings that the mPOA, BNST, MeA, and VMH in FC females had

fewer ERβ-IRs than in MC males (He et al. 2016). Sex differences in ERβ have been reported in the rat mPOA and BNST (Zhang et al. 2002) and mouse BNST (Wolfe et al. 2005; Zuloaga et al. 2014). Since ERβ is involved in a subtle manner in the sexual conduct of males and females, it is required for normal sexual conduct in males displaying delayed ejaculation and in females that exhibit decreased receptivity and attractivity related to an alteration of a volatile chemical signal, most possibly a pheromone (Antal et al. 2012). The ERβ protein is present in the mPOA and BNST that are important for processing of pheromone-induced signals (Antal et al. 2012). This defeminization of sexual conduct is likely mediated by estrogen signaling through ERβ (Scordalakes et al. 2002) and the suppression of typical female responses (Bakker 2003). An increase in ERβ could decrease feminization in females, and ERβ neurons in the mPOA are essential for defeminization (Kudwa et al. 2006). FT females had more ERβ-IRs in the mPOA, BNST, MeA, and VMH than FC females. This expression pattern of ERβ in the four brain regions is similar to that of MC males, indicating that ERβ activation during the neonatal critical period could interfere with the sex-specific organization of the neuroendocrine pathways mediating female reproductive behavior (Sullivan et al. 2011). Thus, FT females reduced lordosis behavior but increased mount behavior. Our conclusion supports the hypothesis that the neonatal presence of estrogen through ERβ caused irreversible masculinization of these structures (Henley et al. 2009). ERβ is introduced in the masculinization of neuroendocrine pathways regulating sex-specific behavior and environmental exposures during critical stages of neuroendocrine development can evoke long term effects on complex behavior (Sullivan et al. 2011). Female rodents possess circuits that control the expression of male-typical mating behavior and their function are normally suppressed by pheromonal inputs (Henley et al. 2010). These circuits may reveal the actions of fetal steroid hormone exposure normally sustained by female rodent species, or it may reflect a sexually monomorphic aspect of neural development that causes the organization of male-typical circuits in both sexes (Baum and Kelliher 2009). We speculate that more ERβ-IRs in the mPOA, BNST, MeA, and VMH in FT females produced the distribution male-like pattern and might be related to reduced lordosis and increased mount behavior. A recent study found that ERβ is not required for the organization and activation of male C57BL/6J sexual conduct (Naulé et al. 2016), but a different study show that neural ERβ deletion alters the timing of pubertal maturation in females (Naulé et al. 2016), suggesting transient prepubertal functions for ERβ in both sexes (Naulé et al. 2016). The discrepancy between these recent studies and our work may be related to species and research factors.

Some research indicates that ERα is primarily accountable for sexual preference and masculinization while ERβ is more important for defeminization (Kudwa et al. 2006; Wersinger and Rissman 2000). On the other hand, it is now well-recognized that the relationship between ERα and ERβ is dynamic and complex. For example, ERβ activation can antagonize ERα-dependent transcription (Matthews et al. 2006; Rissman 2008), but the two ER subtypes can also have synergistic or sequential effects (Rissman 2008). Double knockout of ERα and ERβ eliminated male mouse sexual conduct (Kudwa et al. 2005). ERα and ERβ may interact to regulate male and female sexual conduct (Opendak et al. 2016). Our results show that sexual conduct during partner preference and sexual conduct with intact males or estrous females correlates positively with ERα and ERβ expression levels in the MeA, mPOA, BNST, and VMH.

CONCLUSIONS

Our most robust finding was that E_2 treatment during perinatal development alters female partner preferences and sexual conduct. E_2 appears to have a masculinizing and defeminizing effect. FT females preferred to spend more time with an anesthetized or awake estrous female and less time with an anesthetized or awake sexually active male than did FC females. FT females presented less female sexual conduct, receptivity, proceptivity, and possibly attractivity to males, while occasionally showing ejaculation and mounting patterns when placed with an estrous female. The distribution of ERα was not a completely male-like pattern, more ERα-IRs in the BNST, and MeA, were found in FT females than in MC males, while the distribution of EEβ in the mPOA, BNST, MeA, and VMH was completely male-like. We propose that estradiol treatment during perinatal development alters the ERβ/ERα ratio in different brain regions and plays an important role in the development of male partner preference and sexual conduct.

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Authors' contributions: Feng-Qin HE conceived of and designed the study; Bing YU performed the experiments; Quan-Li XIANG observed behavior and analyzed experimental data; Xiao-Xia CHENG wrote the paper; Zi-Jian WANG reviewed and edited the manuscript. All authors read and approved the manuscript.

Competing interests: All authors report no conflict of interests.

Availability of data and materials: All data generated or analysed during this study are included in this published article.

Consent for publication: The author confirms: that the work described has not been published before; that it is not under consideration for publication elsewhere; that its publication has been approved by all co-authors; that its publication has been approved by the responsible authorities at the institution where the work is carried out.

Ethics approval consent to participate: All study protocols were approved by the Institutional Animal Care and Use Committee, Xi'an University.

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