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Changes of Estrogen in Serum and Estrogen Receptor β in the Relevant Brain Regions Following Mating Behavior of the Male Mandarin Vole *Microtus mandarinus*

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Abstract: In order to investigate the estrogen and estrogen receptor β changes after mating behavior of male mandarin vole (*Microtus mandarinus*), the radioimmunoassay (RIA) and immunohistochemistry methods were used to investigate changes of the serum estrogen (E) concentrations, estrogen immunoreactive neurons (E-IRs) and estrogen receptor β immunoreactive neurons (ERβ-IRs) in the relevant brain regions following mating behavior. Fifteen sexually matured male voles were randomly divided into three groups and treated differently: (1) control group: voles were exposed to clean hard-wood shavings (*n*=5), (2) exposure group: voles were exposed to the soiled bedding for more than 24 h on which estrous females had been placed (*n*=5), and (3) mating group: voles were placed with an estrous female for more than 24 h (*n*=5). The results showed circulating serum E concentrations were significantly higher in the mating group than in the exposure group and the control group, and there were no significant difference between the exposure group and the control group. E-IRs and ERβ-IRs were detected in the following brain regions related to mating behavior: the arcuate nucleus (ARC), bed nucleus of the stria terminalis (BST), lateral septal nucleus (LS), medial amygdaloid nucleus (ME), medial preoptic area (MPO) and ventromedial hypothalamic nucleus (VMH). The results showed that there were significantly more E-IRs in the six brain regions in the mating group than in the control group and the exposure group, and there were no significant difference between the exposure group and the control group except for LS. There was no significant difference in ERβ-IRs in the six brain regions among the three groups, and there were some lighter -stained ERβ-IRs in these brain regions. The results suggested that estrogen affect mating activity of male mandarin voles, but ERβ might not play an important role in mating behavior of male mandarin voles. Instead, it might be through other receptors.

Key words: Mandarin voles (*Microtus mandarinus*)；Estrogen; Estrogen receptor β; Radioimmunoassay; Mating behavior

交配行为对雄性棕色田鼠雌激素及雌激素 β 受体在相关脑区的影响

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摘要：应用行为观察、放射免疫分析和免疫组织化学相结合的方法，研究了雄性棕色田鼠在交配后血清中的雌二醇（E）、与交配行为有关的脑区E免疫阳性细胞数目（E-IRs）、雌激素β受体（ERβ）免疫阳性细胞数目（ERβ-IRs）的变化。将睾丸下降的成年雄性棕色田鼠分成3组：(1) 对照组：嗅闻24 h新鲜锯木。(2) 暴露组：嗅闻24 h动情期雌鼠底物。(3) 交配组：与动情期雌鼠交配24h。 放射免疫检测血清中的E浓度，交配组比暴露组、对照组显著增高，暴露组和对照组无显著差异。通过免疫组化检测与性行为有关的脑区：弓状核(ARC)、终纹床核（BST）、隔外侧核（LS）、杏仁内侧核（ME）、内侧视前区（MPO）、下丘脑腹内侧核（VMH）E-IRs和ERβ-IRs，E-IRs在交配组比对照组和暴露组各区域都显著增多，暴露组比对照组在隔外侧核显著增多外，其他区域无显著差异。

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ERβ-IRs在这3组之间均无显著差异，而且ERβ免疫阳性细胞颜色浅淡。结果表明：雌激素对雄性棕色田鼠的交配活动起一定的作用，但可能通过其他受体，ERβ在雄性棕色田鼠的交配活动中可能未起重要作用。

关键词：棕色田鼠；雌激素；雌激素受体 β；放射免疫分析；交配行为
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Male sexual behavior is mediated in part by androgens, but in several species, mating is also influenced by estrogen (the main estrogen is estradiol) formed locally in the brain (Clancy et al, 2000; Cooper et al, 2000; Cushing et al, 2004). Both testosterone (T) and estrogen (E) are secreted by the testes into the systemic circulation, and T is enzymatically converted centrally and peripherally into E by aromatization (Whalen et al, 1985). Estrogen receptor β (ERβ) offers a new mechanism for E to act in a tissue-specific manner (Kuiper et al, 1996). Previous investigation showed that estrogen receptor α (ERα) might play roles in the regulation of some mammal sexual behavior (Krege et al, 1998; Wersinger et al, 1999). It was found that ERβ gene-disrupted female mice (ERβ-knockout; ERβKO) displayed typical sexual behaviors and could successfully reproduce descendants (Ogawa et al, 1999). However, these mice showed a prolonged sexual receptivity during the estrous cycle contrasted with wild-typed mice (Ogawa et al, 1999). It suggested that ERβ play a subtler role than ERα in the regulation of sexual receptivity (Greco et al, 2003). In female rats, there existed the diversity in expression of type of Estrogen receptors (ERs) in cells of different brain areas after various mating stimuli (received either mounts or intromission). It showed that both ERα and ERβ played roles in the integration of hormonal information and information related to mating stimuli (Greco et al, 2003). Through investigating the relation between ERβ and male mice mating behavior, Temple et al (2003) suggested that ERβ play an important role in the timing of male sexual behavior at puberty by using immunohistochemistry method. Furthermore, they suggested that ERβ regulate ejaculatory behavior. However, Scordalakes et al (2002) established that ERβKO male mice showed almost normal levels of mounts, intromissions and ejaculations. Previous researchers focused more on relation between ER (ERα or ERβ) and sexual behaviors in female rodents, but the function of ERβ in male rodent sexual behavior has not yet been clarified.

Estrogen receptor-containing is widely, but selectively, distributed within a highly interconnected network of nuclei in the limbic system, and it controls sexual behaviors (Krege et al, 1998). The arcuate nucleusi (ARC) may link neurotransmission to ejaculation (Cameron & Erskine, 2003；DeJong et al, 2005); estrogen action in either medial amygdaloid nucleusi (ME) or medial preoptic area (MPO) may facilitate mounting (Huddleston et al, 2003; Clancy et al, 2000); the bed nucleus of the stria terminalis (BST) is probably part of the neural path through which chemosensory information is relayed through the BST, and the integrity of the region appears essential for the display of noncontact erections (Brackett et al, 1984; Kondo et al, 1997); the ventral lateral septum (LS) and ventromedial hypothalamic nucleus (VMH) also serve as key sites for transduction of sexually and behaviorally relevant steroid signals (McGinnis et al, 1996). However, it is not clear whether these brain regions are activated exclusively in response to pheromonal stimulation or they link ERβ to regulate male sexual behavior.

Microtine rodents are an ideal group for comparative studies due to their taxonomic relationship and profound differences in reproductive biology and social organization (Young et al, 2004), and thus are informative for studies on the neurobiological bases of social behavior (Aragona et al, 2006). The mandarin voles have been established to exhibit a monogamous mating system (Tai et al, 2001). In the previous study, it was observed that the decrease of estrogen receptor β immunoreactive neurons (ERβ-IRs) in the ME, MPO, BST, LS and VMH affected male mandarin voles' social recognition and aggressive behavior (He et al, 2004). However, the relation of ERβ in these brain regions and sexual behaviors of male mandarin voles remains unknown. This experiment was designed to systematically compare estrogen immunoreactive neurons (E-IRs) and ERβ-IRs in the ARC, BST, LS, ME, MPO and VMH of gonadally intact male mandarin voles, following mating, exposure soiled estrous female bedding (female pheromones) and exposure clean bedding, and it also investigated how the serum E was affected by using radioimmunoassay (RIA) method.

1 Materials and Methods

1.1 Animals

Twenty five healthy adult mandarin voles (male:

female=15∶10, weight 30-36 g, 90 days old) were obtained from an outbreed colony and reared in the College of Life Science, Shaanxi Normal University, Xi'an, China. The colony of mandarin vole was established in 1997 with wild-captured animals from Lingbao city, Henan Province. The animals were housed individually in clear plastic cages (40cm ×28cm×50cm). The voles were held at a photoperiod of 12L∶12D and temperature at 24-26℃. Hardwood shavings and cotton batting were provided as substrate and bedding. Rabbit chow (Laboratory Animals Center, Xi'an Medical University), carrot and malt were provided *ad libitum* in this experiment. All methods treating voles were approved by the Institutional Animal Care and Use Committee in Shaanxi Normal University.

1.2 Behavior tests

The animals whose bilateral or unilateral testis had descended were randomly divided into three groups: (1) voles were exposed to clean bedding (control group, *n*=5); (2) voles were exposed to the soiled bedding on which estrous females had been placed for more than 24 h (exposure group, *n*=5); and (3) voles were placed in contact with an estrous female in a soiled cage which had not been cleaned for more than 24h (mating group, *n*=5). Female intact voles were brought into estrus with estradiol benzoate (0.00075 mg/g, 24 h before testing) and progesterone (0.015mg/g, 4-6h before testing), and estrous state of the females was monitored by taking vaginal smears. Sniffing and mounting behaviors of all males in the mating group were recorded. Successful matings were indicated by presence of a vaginal plug. Males were individually anesthetized, and the blood sample collection and perfusions were performed 24h after the exposure experiment.

1.3 Blood sample collections and Radioimmuno-assay (RIA)

The blood of three groups was collected by eyeball excised from deeply anesthetized mandarin voles by using sodium pentobarbital (40mg/kg). The serum was carefully separated and collected from blood by using centrifuge, and subsequently serum E concentrations were assayed by RIA using ^{125}I kits purchased from ICN Biochemicals Inc. (Carson, CA) in No. 2 People's Hospital of Shaanxi Province, Xi'an.

1.4 Immunocytochemistry

After the blood samples from three groups were collected, the animals were perfused via the left ventricle with 0.1mol/L phosphate-buffered saline (PBS), 150mL, pH7.2-7.4, which were followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB), 400 mL, pH7.2-7.4. The voles were decapitated. Their brain tissues were removed carefully and placed back into the fixative at 4℃ in a refrigerator for 3h, and then stored in 30% sucrose until they sank to the bottom of the container.

Coronal sections 40μm from olfactory bulb to the anterior part of the pons were sectioned on a freezing sliding microtome. Free-floating sections were divided into three parts and rinsed in 0.01mol/L PBS at the same time. All sections were blocked with normal goat serum (Boster Company) for 30 min at 37℃ to inactivate exogenous peroxidase, followed by three times rinsing in 0.01mol/L PBS (pH7.4) for 5min each. Two parts of the all sections were respectively incubated in a rabbit polyclonal E primary antibody (8∶100, E2880, Sigma) and a rabbit polyclonal ERβ (1∶100, Sc-8974, Santa cruz) primary antibody for 48h at 4℃. Having been rinsed in PBS, the sections were further incubated with the goat anti-rabbit secondary antibody (Boster Company) for 30min at 37℃, and then rinsed in PBS. The sections were finally incubated with strept avidin-biotin complex (SABC, Boster Company) for 30 min at 37℃. After being rinsed in PBS, the sections were incubated in a chromogen solution containing 0.03% 3,3'-diaminobenzadine (DAB, Boster Company) for about 10-30min at room temperature. Finally, the sections were mounted onto subbed glass slides and air-dried overnight. The sections were then dehydrated in ascending ethanol solutions, cleared in xylene, cover-slipped with DePeX and observed with an Olympus light microscope. Negative controls were carried out for other sections by using the same procedure, in which 0.01mol/L PBS was used instead of primary antibody.

E-IRs and ERβ-IRs in the ARC, BST, LS, ME, MPO and VMH were observed in the vole brains of three groups. The brain regions were discriminated referring to the illustrative plates of rat brain (Bao & Su, 1991) and the previous brain sections. In order to balance the different colors of different slides due to different staining date or time, three medium stained sections of each brain region were chosen from each animal. The cell shape in these brain sections was unregulated. The cell diameter size was 2-10μm. There was significant difference between a dark dirt mark and a real cell: a dark dirt mark was bigger and strongly-stained than a cell. Slides were coded and the person counting the immunoreactive cells was blind with the treatment group. The number of positive cells was

counted randomly in per standard area (60μm×60μm) by using a grid sampling in the three medium stained sections of the same animal under an Olympus visible light microscope, not was counted by the levels or intensity of E and ERβ immunoreactive labeling. The researchers averaged the data from the three medium staining sections and then conducted subsequent analysis.

1.5 Quantitative analysis

SPSS (Statistical Package for Social Science) 10.0 was used for data analysis. Because serum E concentrations did not distribute normally, the Skrual-Wallis test which was followed by post hoc tests of Mann-Whitney *U* tests was used to analyze and compare the differences of serum E concentrations among the three groups. If $P<0.05$, it indicated significant difference between various groups, and E-IRs and ERβ-IRs in the ARC, BST, LS, ME, MPO and VMH distributed normally. Data were assayed with a One-way factorial analysis of variance (ANOVA). If $P<0.05$, it was then followed by post hoc tests to make a comparison with Fisher's LSD test (Apostolinas et al, 1999).

2 Results

2.1 The changes of the serum E concentrations by RIA method

Through testing the serum E concentrations in the three cases by using RIA (mean±SE) respectively, it was found that there were significant differences among them by Skrual-Wallis test ($P<0.05$). The serum E concentration of mating group (280.1697±57.9595 ng/dL) was significantly higher than those of control group (35.4000±25.9962 ng/dL) and exposure group (62.4667±34.3015 ng/dL). The control and exposure group showed no differences although the former was fewer than latter. However, the mating group showed significant differences from both the control group and exposure group (Fig.1). It was followed by Mann-Whitney *U* tests, and the results were the same as in Skrual-Wallis test.

2.2 The number of estrogen immunoreactive neurons and estrogen receptor β immunoreactive neurons in six brain regions in control, exposure and mating groups

In comparison with the control group, the number of E-IRs of the exposure group was not significantly different in these brain areas except for the LS ($P>0.05$). The number of E-IRs in the mating group was significantly more than in the control and the exposure groups in these brain regions ($P<0.05$)(Tab. 1, Fig.2).

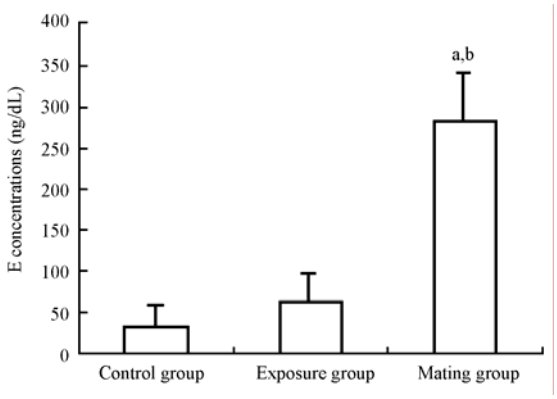

Fig. 1 Mean of circulating serum E concentrations（ng/dL） measurements by RIA

a: Between serum E concentrations of mating and control groups have significant difference by the non-parametric test of Skrual-Wallis tests, which was followed by post hoc test of Mann-Whitney U test, $P<0.05$; b: Between serum E concentrations of mating and exposure groups have significant difference by the non-parametric test of Skrual-Wallis tests which was followed by post hoc test of Mann-Whitney U test, $P<0.05$.

The number of ERβ-IRs was not significantly different in the six brain regions among the control, expose and mating groups (Fig.2) ($P>0.05$). In addition, the number of ERβ-IRs was fewer and lighter-stained than E-IRs in the three groups(Tab. 2).

3 Discussion

The present study first established that circulation of serum E concentrations in male mandarin vole following mating were significantly higher than in male mandarin vole exposed to bedding of opposite sex. Furthermore, E-IRs in six brain regions that were involved in mating behavior were significantly higher. It may be inferred that estrogen may be involved in copulatory behavior of male mandrain voles. The previous research found that the brain regions of males containing cell bodies and/or fibers of gonadotropin releasing hormone (GnRH) neurons might be activated through somatosensory stimuli (mounts, intromissions and ejaculations) and chemosensory input via olfactory bulb, causing a possible increase both in the serum T concentrations and brain T during the copulation of male-female individuals. T is enzymatically converted centrally and peripherally into E by aromatization (Whalen et al, 1985). Therefore, both the serum E concentrations and brain E were inducted to increase. On the other hand, serum E concentrations might also be increased in mating behavior through hypothalamic-pituitary-adrenal axis

Tab. 1 Total number of estrogen immunoreactive neurons (mean±*SE*) in per standard area of each brain region in the control, exposure and mating groups (*n*=5)

Brain region	Control group（*n*=5）	Exposure group（*n*=5）	Mating group（*n*=5）
ARC	3.3±0.434	4.5±0.545	7.5±0.576[a,b]
BST	4.6±0.702	5.0±0.890	8.3±1.174[a,b]
LS	23.4±2.182	29.7±1.700[c]	39.0±15.666[aa,b]
ME	12.5±4.532	13.0±5.014	26.4±11.216[a,b]
MPO	8.9±3.254	10.5±4.033	19.1±11.630[a,b]
VMH	16.4±2.204	17.5±3.176	23.5±11.005[a,b]

ARC: arcuate nucleus. BST: bed nucleus of the stria terminalis. LS: lateral septal nucleus. ME: medial amygdaloid nucleus. MPO: medial preoptic area. VMH: ventromedial hypothalamic nucleus. Data are expressed as Mean±*SE* and analyzed by post-hoc test. a: indicates significant difference between control and mating group, $P<0.05$. aa: indicates greatly significant difference between control and mating group, $P<0.01$. b: indicates significant difference between exposure and mating group, $P<0.05$. c: indicates significant difference between control and exposure group, $P<0.05$.

Tab. 2 Total number -of estrogen receptor β immunoreactive neurons（mean±*SE*） in per standard area of each brain region in control, exposure and mating groups（*n*=5）

Brain region	Control group（*n*=5）	Exposure group（*n*=5）	Mating group（*n*=5）
ARC	4.8±3.144	4.4±0.879	4.1±1.294
BST	2.2±0.207	2.5±0.547	3.2±0.816
LS	2.2±0.960	2.3±1.581	2.5±0.894
ME	2.3±1.239	2.7±2.073	2.5±0.273
MPO	3.7±1.186	3.9±1.303	3.5±1.923
VMH	7.3±2.204	7.5±0.917	8.5±1.800

ARC: arcuate nucleus. BST: bed nucleus of the stria terminalis. LS: lateral septal nucleus. ME: medial amygdaloid nucleus. MPO: medial preoptic area. VMH: ventromedial hypothalamic nucleus. Data are expressed as Mean±*SE* and analyzed by post-hoc test.

regulating adrenal zone reticularis secreting estrogen from the adrenal gland (Williams et al, 2001). The conclusion is consistent to the previous findings that not only androgen is the gonadal steroids that play a role in mediating male copulatory behavior in rats, but also estrogen is necessary for the restoration of male copulatory behavior (Vagell & McGinnis, 1997; Greco et al, 2003). The previous research also found that E increases olfactory investigation during the rats noncontact test, and maintains intromission patterns and ejaculatory behavior (Greco et al, 2003). However, aromatization of testosterone to estrogen is not necessary for normal mating behavior in Syrian hamsters (Cooper et al, 2000). It can be conclude that the different results are due to the different species. The present study indicated that the serum E concentrations of male mandarin voles, after being exposed to female soiled bedding, were more than the control group, but it was not significant. E-IR neurons also showed significant difference in all these brain regions except for the LS, suggesting that the LS might be specific to phermonal detection. It might be the influence of pheromones from the soiled bedding of estrous female through olfactory bulb, which induce the serum E concentrations to be increased. However, the sensitivity of E in different brain regions might not be alike, so E-IR distribution in different brain regions were different. This inference was clarified by determinating the stimulation of pheromones and may have a certain effect on mating behavior of male mardarin voles.

In the present experiment, ERβ-IRs in six brain regions were not increased either following exposure to female soiled bedding or following mating with estrous females. It is inferred that ERβ might not affect mating behavior of male mandarin vole. The conclusion is coincided with the findings that the ARC, LS, and MPO show much fewer stains for the ERβ subtype in guinea pig (Warembourg & Leroy, 2004). The lack of ERβ-IR

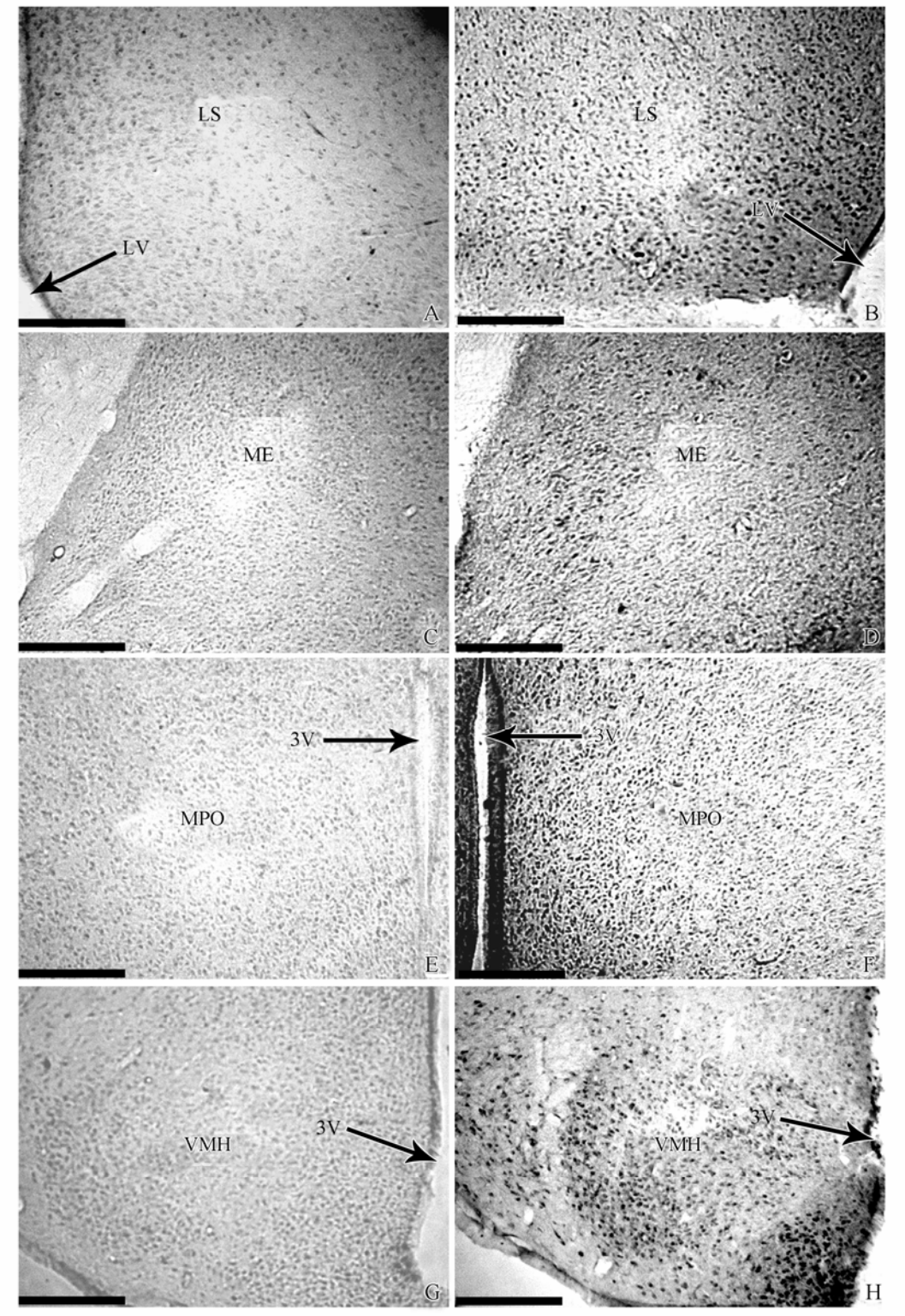

Fig. 2 ERβ and E immunoreactivity of *Microtus mandarinus*

A: ERβ control group in the lateral septal nucleus; B: E mating group in the lateral septal nucleus; C: ERβ control group in the medial amygdaloid nucleus; D: E mating group in the medial amygdaloid nucleus; E: E control group in the medial preoptic area; F: E mating group in the medial preoptic area; G: E control group in the ventromedial hypothalamic nucleus; H: E mating group in the ventromedial hypothalamic nucleus.
3V: Third ventricle; VMH: Ventromedial hypothalamic nucleus; LS: Lateral septal nucleus; LV: Lateral ventricle; ME: Medial amygdaloid nucleus; MPO: Medial preoptic area. Scale bar=100μm.

in the ARC has also been found in the sheep (Scott et al, 2000), rat (Li et al, 1997; Shughrue & Merchenthaler, 2001) and mouse (Mitra et al, 2003). ERβKO mice can display typical sexual behaviors and can successfully reproduce, which ERαKO mice can not display typical sexual behaviors and can not successfully reproduce (Temple et al, 2003). It hints that through α receptor estrogen regulates amount of nitric oxide synthase (NOS), and influences release of Dopamine blockade. It also hints that male mice sexual behaviors are led through Dopamine blockade blocks action of Dopamine (Scordalakes et al, 2002). This result may extend the hypothesis that ERβ might not mainly regulate sexual behaviors of most male mammals.

Though the number of ERβ-IR neurons of these brain regions in mating and expose groups did not increase, the circulating of serum E concentrations and E-IR neurons in these brain regions were increased significantly. The previous study confirmed that estrogen might cause an early rise of testosterone by modifying the sensitivity of Hypothalamic-Pituitary-Gonadal (HPG) axis, and induce androgen receptor (AR) activity to be improved (Temple et al, 2000). During male mandarin vole mating experiment, AR immunoreactivity (AR-IR) in these brain areas relevant to mating behavior were significantly increased (unpublished results). Though the distributions of androgen and estrogen receptors in brain regions are parallel, functions of the two forms of receptors to central nervous system are opposite (Xu et al, 1999). Male prairie voles express more AR than females in the MPO, VMH, ventrolateral portion of the hypothalamus, BST and ME, and express less estrogen receptor than females in these brain regions following sociosexual behaviors (Cushing et al, 2004). Consequently, estrogen receptor may mainly regulate female sex behaviors. The activation of estrogen receptors in males is necessary for the restoration of some behaviors. However not all sociosexual behaviors, androgen and estrogen may play a role in the integration of the restoration of male copulatory behavior (Greco et al, 1998).

The hypothalamic regions contain both ERα and ERβ subtypes with regional variations (Warembourg & Leroy, 2004). One role of ERβ is to modulate ERα transcriptional activity and thus the relative concentration of each subtype will be a key determinant of the cellular responses to estrogenic ligands (Zhang et al, 2001). The differential regional organization of ERα and ERβ elicits different functional effects in one area and in receptor-dependent manner. ERα predominates in the MPO and the ARC. The two regions are important in mediating the reproductive functions and sexual behaviors. The low level of immunolabeling for ERβ suggests that ERβ, unlike ERα, may play only a limited role in the estrogenic regulation of these functions (Warembourg & Leroy, 2004). However, the function of ERα and ERβ are different through studying various mammals: Male prairie voles in sociosexual behaviors expresses fewer ERα in variety of brain regions, showing ERα is not important in sociosexual behaviors in monogamous voles (Greco et al, 1998); in the rat and hamster, ERα is advantageous to mating behavior, and decrease of ERβ may induce the increase of ERα (Zhang et al, 2001). In addition, the study suggests that estrogen regulates social behavior in other way (Fugger et al, 2000). In the present experiment, both of serum E concentrations and E-IR in ARC, BST, LS, ME, MPO and VMH were significantly increased during mating behavior, but ERβ-IR neurons were not increased in these brain regions. It need to be investigated in future studies to clarify whether ERα are involved in the mating behaviors of male Mandarin voles by using other direct neurobiological method.

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