Seasonal changes of androgen receptor, estrogen receptors and aromatase expression in the medial preoptic area of the wild male ground squirrels (*Citellus dauricus* Brandt)

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Abstract

The wild ground squirrel is a typical seasonal breeder. In this study, using RT-PCR, western blot and immunohistochemistry, we investigated the mRNA and protein expressions of androgen receptor (AR), estrogen receptors α and β (ERα and ERβ) and aromatase cytochrome P450 (P450arom) in the medial preoptic area (MPOA) of hypothalamus of the wild male ground squirrel during the breeding season (April), the non-breeding season (June) and pre-hibernation (September). AR, ERα, ERβ and P450arom protein/mRNA were present in the MPOA of all seasons detected. The immunostaining of AR and ERα showed no significant changes in different periods, whereas ERβ and P450arom had higher immunoreactivities during the breeding season and pre-hibernation when compared to those of the non-breeding season. Consistently, both the protein and mRNA levels of P450arom and ERβ were higher in the MPOA of pre-hibernation and the breeding season than in the non-breeding season, whereas no significant difference amongst the three periods was observed for AR and ERα levels. These findings suggested that the MPOA of hypothalamus may be a direct target of androgen and estrogen. Androgen may play important regulatory roles through its receptor and/or the aromatized estrogen in the MPOA of hypothalamus of the wild male ground squirrels.

Introduction

Estrogen and androgen hormones play important roles in brain sexual differentiation and sexual behavior of the vertebrates.[1] The effects of androgens are mediated by androgen receptor (AR) to regulate target gene tran-scription.[2] In brain, AR is closely related to male reproductive behavior, and especially in wild seasonal breeding animals AR often has emerged in seasonal variation.[3] In addition, androgens can be converted to estrogens by aromatase cytochrome P450 (P450arom), which could potentially change the local estrogen levels.[4] P450arom is found in various tissues in both females and males, thus estrogens are produced not only in gonads but also in extra-gonadal localizations such as bone,[4] brain,[5] adipose tissue,[6] breast,[7] and skin.[8] In brain, by regulating the local estrogen levels, P450arom participates in the sexual differentiation[9] of brain regions involved in the control of gonadotropin secretion and sexual behavior.[10]

By binding to its two nuclear estrogen receptors, estrogen receptor alpha (ERα)[11] and beta (ERβ),[12] estrogen influences a wide range of biological activities, which is not merely restricted to the development of the reproductive and endocrine systems.[13,14] In brain, ERα and ERβ are abundantly expressed in the hypothalamus.[15] The expression levels of ERα and ERβ in hypothalamus are closely related to the reproductive status.[16] AR and P450arom are highly expressed in hypothalamus and limbic system in mammals as well.[15,17,18] In the medial preoptic area (MPOA) of hypothalamus, a reproduction-related area that primarily controls male sexual behavior,[19,20] co-expression of AR, ERs and P450arom has been found.[15,17,18] Meanwhile, the expression of P450arom in the MPOA indicates that androgens may as well be converted into estrogens to regulate hypothalamic function and male sexual behavior. The MPOA P450arom expression and its involvement in the regulation of reproductive behavior of many vertebrates has been demonstrated, including mammals,[21] birds,[22] reptiles[23] and fish.[24]

The wild ground squirrel (*Citellus dauricus* Brandt) is a typical long-day seasonal breeder whose annual life cycle can be roughly divided into the breeding season (April to May), the non-breeding season (June to September) and hibernation (October to the following March). Our previous studies have found that testicular morphology and function of the wild male ground squirrels gone through robust changes throughout the year, which correlate nicely with the expression profiles of AR, ERs and P450arom.[25] Interestingly, unlike other known seasonal breeding rodents, the wild ground squirrels in September, namely pre-hibernation, already show a rise in testosterone level from the non-breeding season, accompanied by revitalized spermatogenesis.[26,27] To understand the potential impact of androgens and estrogens on hypothalamic function, the present study investigated the expressions of AR, ERα, ERβ and P450arom in the MPOA of the male wild ground squirrels during the breeding season, the non-breeding season and pre-hibernation.

Materials and Methods

Animals

The wild male ground squirrels that were regarded as adults according to their body weights (242-412 g) were captured on September 27th of 2013 in the pre-hibernation period (n=18), on April 20th after emergence from hibernation in the breeding period (n=15) and on June 9th of 2014 in the non-breeding period (n=16) in Hebei Province, China.

All the procedures on animals were carried out in accordance with the Policy on the Care and Use of Animals by the Ethical Committee, Beijing Forestry University and approved by the Department of Agriculture of Hebei province, China (JNZF11/2007). For the brain samples for immunohistochemistry, wild ground squirrels were weighed and deeply anesthetized with sodium pentobarbital solu-

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tion (30 mg/kg) and then perfused transcardially with 150 mL of 0.9% saline, followed by 250-300 mL of 4% paraformaldehyde (37°C) in 0.1 M PBS (pH 7.3). Then the brain samples were postfixed for 12 h at room temperature in 4% paraformaldehyde and cryoprotected in 20% sucrose in 0.1 M PBS and stored at 4°C until processed. Brain sections of the MPOA were cut coronally into 7-10 μm for immunohistochemistry.[28,29] Otherwise the brain samples were dissected, snap frozen in liquid nitrogen and then kept in -80°C for protein and mRNA detections.

Immunohistochemistry

The serial sections of brain tissues were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with primary antibodies (1:1000) raised against rabbit polyclonal anti-AR (sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-ERα (sc-542, Santa Cruz Biotechnology), rabbit polyclonal anti-ERβ (sc-8974, Santa Cruz Biotechnology) and rabbit polyclonal anti-aromatase (ab18995, Abcam, Cambridge, MA) for 12 h under 4°C. The sections were then incubated with a secondary antibody, goat anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using rabbit ExtrAvidin™ Peroxidase staining kit (Sigma Chemical Co., St. Louis, MO, USA) was performed, followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 Mol Tris-HCl buffer, pH 7.6, plus 30 μl H_2O_2. The specificity of AR, ERα, ERβ and P450arom antibodies has been described in our previous studies.[25,30] The control sections were treated with normal rabbit serum instead of the primary antisera.

Immunohistochemistry score

The score assay protocol for immunohistochemistry was adapted from previous reports.[31,32] In general, the immunohistochemical localization was scored in a semiquantitative fashion incorporating both the intensity and distribution of specific staining. The intensity of specific staining was characterized as no present (0), weak but detectable above control (1), distinct (2), and very strong (3). For each observed tissue component, a summary value we refer to as H-Score was calculated. This consists of a sum of the percentages of positively stained cells multiplied by a weighted intensity of staining H-Score=ΣP_i(i + 1), where i is the percentage of stained cells in each intensity category, and i is the intensity for i = 1, 2, 3. A total H-Score for the tissue section was derived as the sum of the component H-Scores weighted by the fraction of each component observed in the tissue section.

Western blotting

Tissue extraction was done *via* 50 μm coronal slices on a cryostat, where tissue punches (1 mm in diameter) were taken bilaterally throughout the MPOA, and stored in at -80ºC.[28,29] Tissues was homogenized in a homogenizer containing 300 μL of 10 mg/mL PMSF stock and incubated on ice for 30 min to maintain the temperature at 4°C throughout all the procedures. Following centrifugation at 12,000×g for 10 min at 4°C, the supernatant was collected. Protein extracts (25 μg) were mixed with an equal volume of 2× Laemmli sample buffer. Equal amounts of each sample were loaded and run on a 12% SDS-PAGE gel at 18 V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). Membranes were blocked with 3% BSA for 1 h at room temperature. The membranes were washed and incubated with a 1:1000 primary antibody for overnight. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 60 min. Finally, the membrane was washed in 25 ml TBS-T buffer (0.02 M Tris, 0.137 M NaCl and 0.1% Tween-20, pH 7.6) plus 3 μl H_2O_2 and checked with Odyssey infrared imaging system. Preabsorptions of the antibodies were performed with an excess of relative antigens (Sigma) for the negative control. β-actin was selected as the loading control. The bands were quantified using Quantity One Software (Bio-Rad Laboratories, Inc., ver. 4.5) and the related expression ratios to β-Actin were calculated.

RT-PCR

The first-strand cDNA from total RNA was synthesized using StarScript II Reverse Transcriptase and Oligo (dT)18 by TIANScript RT Kit (Tiangen, Beijing, China). The 20 μL of reaction mixture contained 3 μg of total RNA, 1 μL of Oligo (dT)18, 1 μL of 10mM deoxyribonucleoside triphosphate (dNTP), 4 μL of

250 mM Tris–HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$, 2 μL of 0.1 M dithiothreitol, 0.5 μL of RNase Inhibitor and 200 U of StarScript II enzyme. The 25 μL of reaction mixture contained 2 μL of first-strand cDNA, 0.5 μM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 mM Tris–HCl (pH 8.4) and 2.5 U of Taq polymerase (Tiangen). The amplification was under the following condition: 94°C for 3 min for the initial denaturation of the RNA/ cDNA hybrid, 35 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 1 min with a final extension of 10 min at 72°C. The first-strand cDNA was used for PCR amplification with the following primers (Table 1). The PCR product was electrophoresed in the 1% agarose gel and individual bands were visualized by ethidium bromide (EB) staining. The housekeeping gene *Actb* was selected as the endogenous control. The bands were quantified using Quantity One software and the related expression relative to *Actb* were calculated.

Statistical analysis

Statistical comparisons were made with the one-way ANOVA followed by Turkey's test. A value of P<0.05 was considered indication of statistical significance.

Results

Immunolocalization of AR, ERα, ERβ and P450arom in the MPOA of hypothalamus

Immunohistochemical staining of AR, ERα, ERβ and P450arom was performed in the MPOA of the male wild ground squirrels during the breeding season, non-breeding season and pre-hibernation (Figure 1). Strong positive signal of AR was localized in the nucleus in the MPOA throughout all periods (Figure 1 a-c). Similar observations were seen for ERα and ERb (Figure 1 d-i). P450arom was present in the cytoplasm of the MPOA of the breeding

Table 1. Oligonucleotide primers used for RT-PCR.

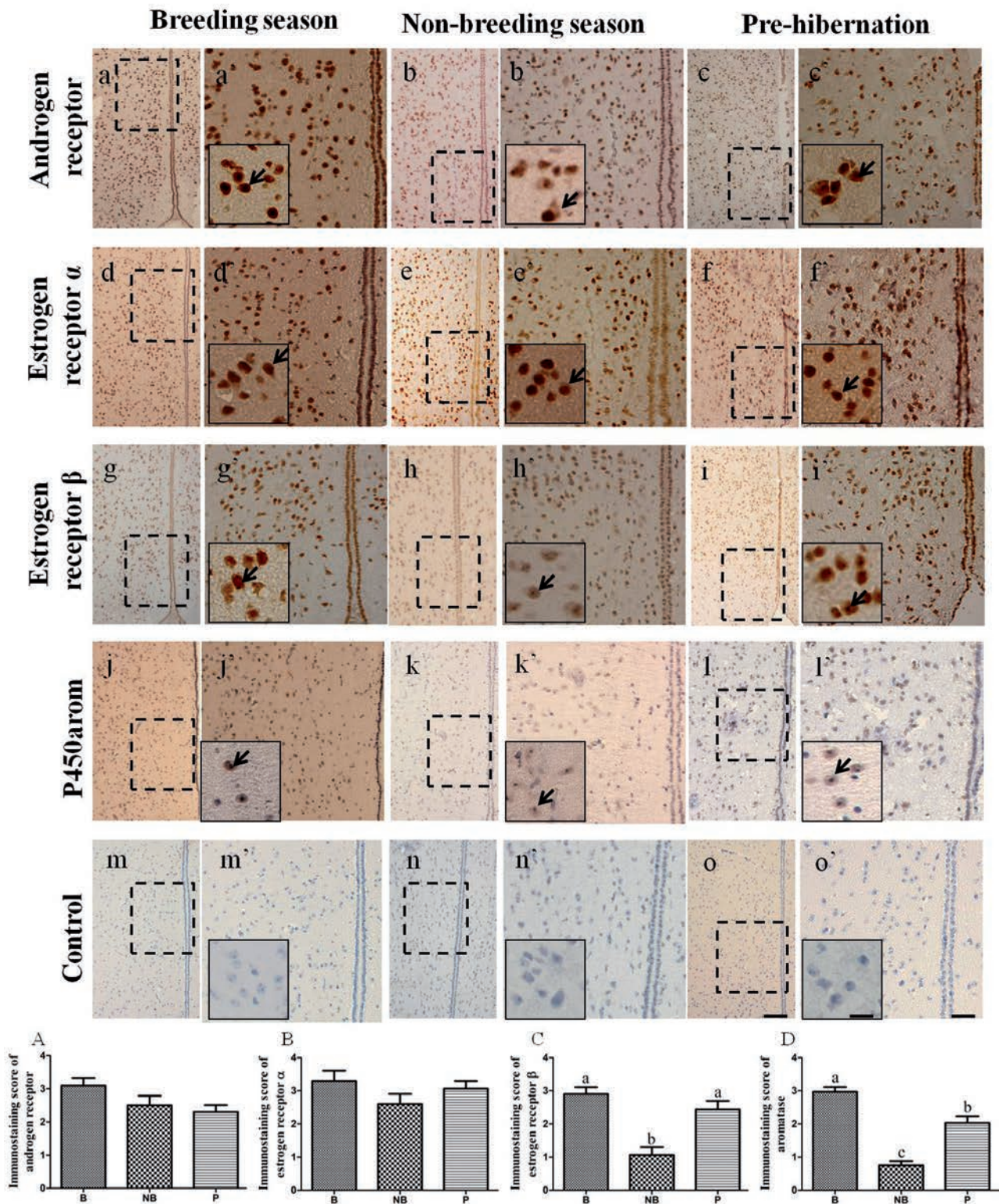| Gene	Sequence of primer (5'-3')	Product size (bp)
AR	F: CATGGCAAACACCATGAGTC R: ATGTCCTGGAAGCCATTGAG	224
ER	F: TGCACCATTGACAAGAACCG R: TCCAGGAGCAAGTTAGGAGC	541
ER	F: TCTGGGTGATTGCGAAGAGT R: CCCCGAGATTGAGGACTTGT	215
CYP19	F: GAGAAAGGCATCATATTTAACA R: CCAAGAAATCTTAAAGAAGATG	333
β-actin	F: GACTCGTCGTACTCCTGCTT R: AAGACCTCTATGCCAACACC	223

Figure 1. Immunohistochemistry of AR, ER α, ERβ and P450arom in the MPOA of hypothalamus of the wild male ground squirrels during the breeding season, the non-breeding season and pre-hibernation. x' is the magnification of the dashed-line square in x. In the boxed area on the bottom left in x', immunoreactive cells (arrow) are shown at higher magnification. Bar in x, 100 μm; bar in x', 40 μm; bar in the boxed area, 20 μm (x applies from a to o). The left column (a, d, g, j, m) represents staining in the breeding season; the center column (b, e, h, k, n), and the right column (c, f, i, l, o) represent immunostaining in the non-breeding season and pre-hibernation, respectively. The immunopositivity for AR (a-c), ERα (d-f) and ERβ (g-i) was observed in the nucleus, while the immunopositivity for aromatase (j-l) was in the cytoplasm. Negative controls (m-o) were counterstained with haematoxylin. The immunostaining score of AR (A), ERα (B), ERβ (C) and P450arom (D) showed the differences during the breeding season, the non-breeding season and pre-hibernation. B, the breeding season; NB, the non-breeding season; P, pre-hibernation. Bars represent means ± SD for five independent experiments. Means within the columns marked with different letters indicate significant difference (P<0.05).

season, the non-breeding season and pre-hibernation (Figure 1 j-l). No signal was observed in the negative controls (Figure 1 m-o).

Expression of AR, ERα, ERβ and P450arom proteins

Western blot analysis of proteins extracted from the MPOA of hypothalamus tissues revealed the immunoreactivities of AR, ERa, ERb and P450arom proteins positioned at 101 kDa, 66 kDa, 59 kDa and 55 kDa in different seasons, respectively (Figure 2). The results were normalized to the expression level of β-actin. While AR and ERa expression levelsshowed no significant difference amongst the three seasons (Figure 2 A,B), the expression of P450arom and ERb in the breeding season and pre-hibernation were significantly higher than the non-breeding season (Figure 2 C,D).

Expression of AR, ERα, ERβ and P450arom mRNA

AR, ERa, ERb and P450arom mRNA levels were also detected in the MPOA of the wild male ground squirrels during the breeding season, the non-breeding season and pre-hibernation (Figure 3). In keeping with the protein levels, AR and ERα mRNA expressions remained relatively stable throughout the breeding season, the non-breeding season and pre-hibernation (Figure 3 A,B). The expressions of ERβ and P450arom genes peaked in the breeding season, markedly dropped during the non-breeding season, and then showed a marked increase in pre-hibernation (Figure 3 C,D). After obtaining the sequence of each PCR product, we blasted with the known mRNA sequences of rat, mouse, human and bovine, found the homologous sequence fragments in each species and compare for homology (Table 2).
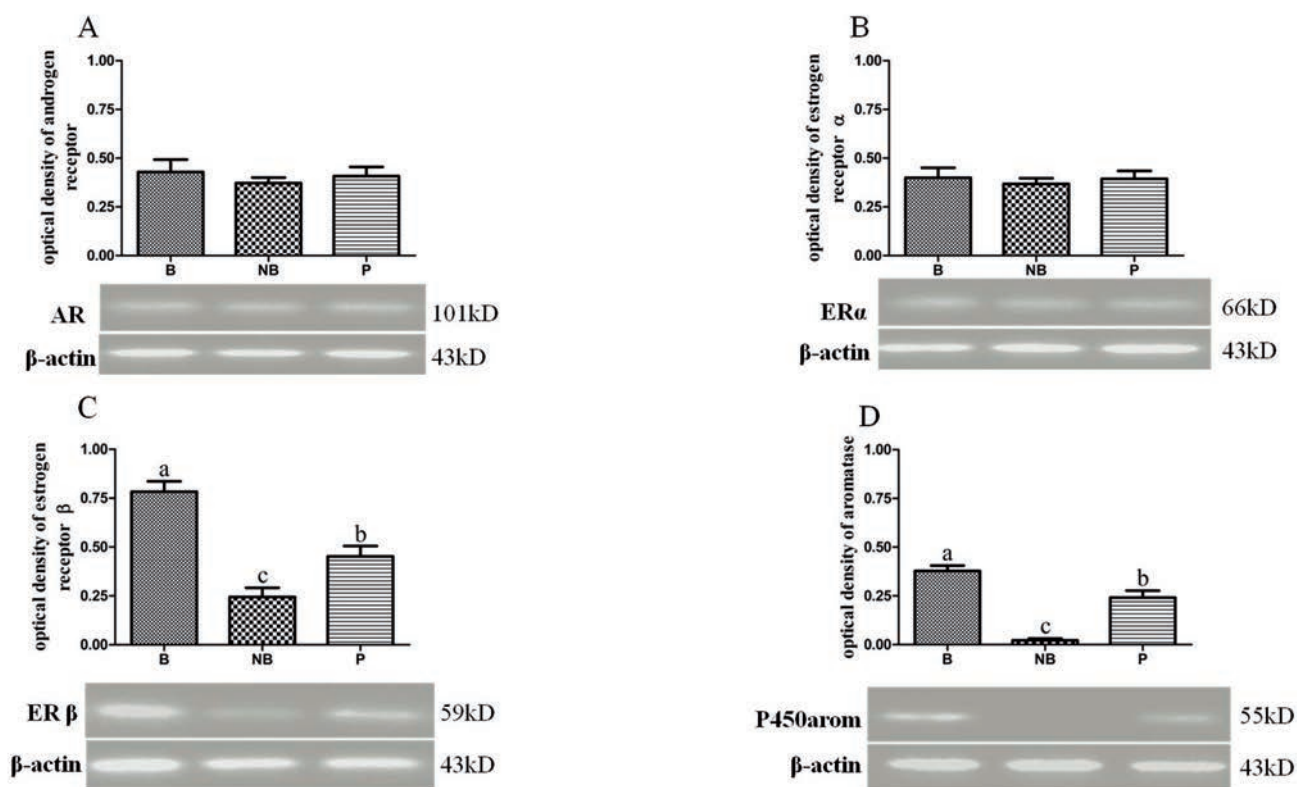

Figure 2. Western blot analysis of the protein level of AR, ERα, ERβ and P450arom during the annual cycle. The expressions of AR (A), ERα (B), ERβ (C) and P450arom (D) showed the changes during the breeding season, the non-breeding season and pre-hibernation. B, the breeding season; NB, the non-breeding season; P, pre-hibernation. Bars represent means ± SD for five independent experiments. Means within the columns marked with different letters indicate significant difference (P<0.05).

Table 2. Nucleotide sequence identity in testis of wild ground squirrel in comparison with rat, mouse, human and bovine.

	Rat (%)	Mouse (%)	Human (%)	Bovine (%)
AR	91.35	90.12	92.31	90.12
ER alpha	85.50	86.68	89.05	89.34
ER beta	93.54	92.74	87.91	84.68
CYP19	78.77	81.01	82.12	83.05
Beta-actin	90.01	91.16	88.37	83.62

Discussion

This was the first study to investigate the expression of AR, ERa, ERb and P450arom in the MPOA of the wild male ground squirrels, which clearly demonstrated the presence of AR, ERa, ERb and P450arom in the MPOA of this wild rodent during the breeding season, the non-breeding season and pre-hibernation. These findings strongly suggested that the hypothalamic MPOA may be a direct target of androgens and estrogens; and estrogens may play important regulatory roles in hypothalamic function in an autocrine/paracrine manner in this species.

The MPOA is a well-known androgen-responsive region that regulates homeostasis, neuroendocrinology and instinctive behavioral systems.[33] Androgens play a pivotal role in the regulation of male sexual behaviors and aggressive behavior at least *via* AR in the MPOA and anterior hypothalamic areas.[34,35] Previous studies showed that the expression levels of AR in the MPOA varied between differ-ent rodents.[36] For example, AR expression in the mouse brain was shown to be much stronger than that of the rat brain, which may explain some of the behavioral differences.[36] As a typical seasonal breeder, the wild ground squirrel exhibits distinct reproductive behavior from season to season. However, we observed a stably high AR expression in the MPOA throughout all seasons. A previous study in Siberian hamster linked photoperiod and the action of androgens, where they also showed that day length fluctuations could induce the expression of AR and steroid receptor coactivator-1.[37] Therefore, the little change in day length (13-15 h) from the breeding season to pre-hibernation in latitudes (N41°14'33"-41°56'55") where the wild ground squirrels habitate may be a possible explanation. On the other hand, the stably strong expression may highlight the indispensable role of AR in normal hypothalamic function maintenance.

P450arom, an enzyme converting the androgens to estrogens, has been found in many tissues, including non-reproductive organs in male.[22-24] In this study, we profiled the seasonal expressions of P450arom in the MPOA of the wild male ground squirrels, where highest protein and mRNA levels of P450arom were observed in the breeding season. The expression of P450arom in brain was shown to be a critical step for maintaining the male sexual behavior.[38] Male mice with *P450arom* knockout in brain exhibited decreased fertility and reduced male sexual behavior.[38] In several birds studied, P450arom mRNA in the MPOA of the non-breeding season was significantly lower than that of the breeding season.[3,22] For example, in the free-living male song sparrow (*Melospiza melodia morphna*), P450arom mRNA expression in the MPOA was higher during breeding season than non-breeding season or molt, which may be owing to the involvement of these brain areas in estrogen-dependent regulation of male sexual behavior.[22,39] Interestingly, in pre-hibernation, P450arom activity in testes had a slight increase, which may be related to the change of reproductive status in this species. Our previous study in the wild ground squirrel
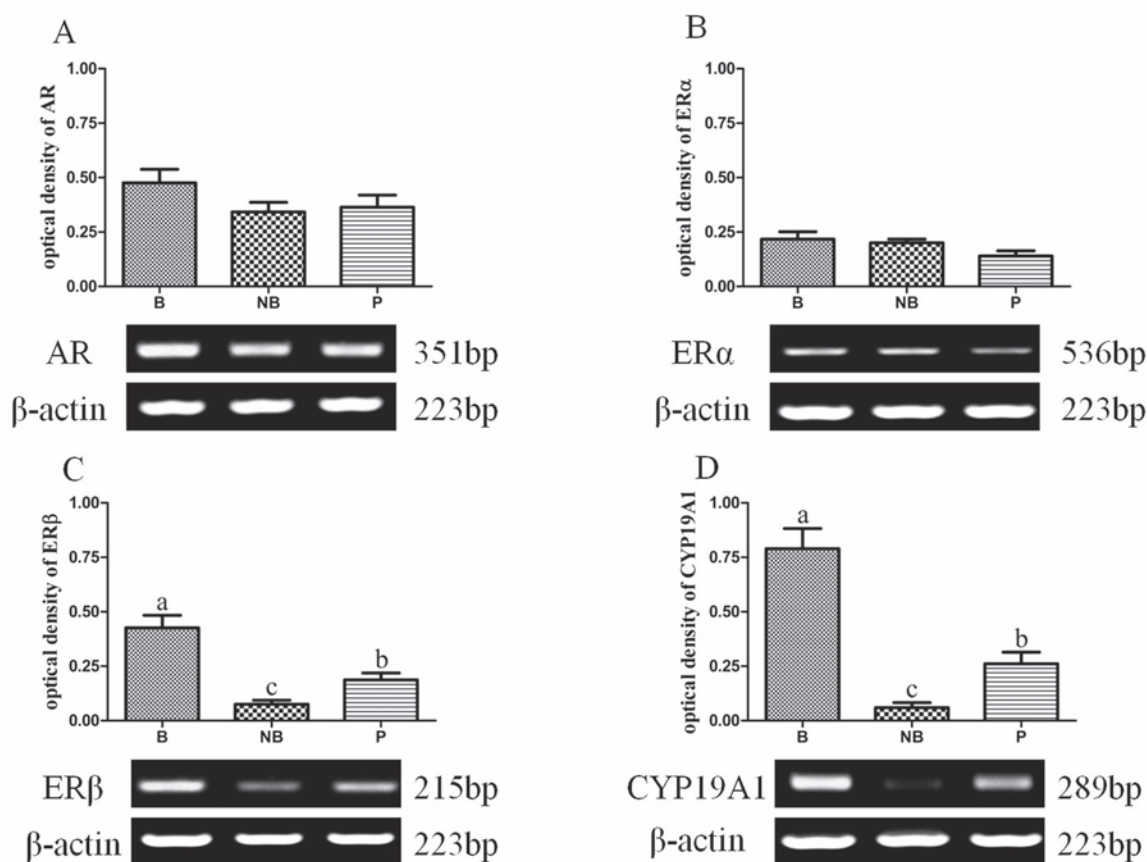

Figure 3. RT-PCR analysis of the mRNA level of AR, ERα, ERβ and P450arom during the annual cycle. The expressions of genes *AR* (A), *ERα* (B), *ERβ* (C) and *CYP19* (D) showed the changes during the breeding season, the non-breeding season and pre-hibernation. B, the breeding season; NB, the non-breeding season; P, pre-hibernation. Bars represent means ± SD for five independent experiments. Means within the columns marked with different letters indicate significant difference (P<0.05).

revealed a testicular recrudescence during pre-hibernation, and P450arom mRNA and protein levels were concomitantly elevated relative to the non-breeding season.[25] The present study showed that P450arom immunoreactivity in the MPOA was positively correlated with reproductive status, suggesting that local aromatization of androgens may be required for the seasonal function of the MPOA neurons.

ERα and ERβ are encoded by separate genes in the male reproductive tract or non-reproductive organs.[40-42] Both ERα and ERβ have been observed in neurons and glia in the brain, and both are expressed throughout the brain with distinct patterns in different brain regions and with differing levels of expression during development.[43] The present results showed that the expression level of ERα remained high throughout all periods, whereas ERβ showed higher immunoreactivity during the breeding season and pre-hibernation compared to the non-breeding season. These findings suggested that the MPOA of hypothalamus may be a direct target of estrogen. Previous studies have reported the relationship between the expression of ERα and ERβ in the MPOA and sexual behavior in some seasonally breeding avian species. However, the seasonal changes of ERα and ERβ are often modest and inconsistent across studies. For instance, there was no detectable seasonal change in either ERα or ERβ mRNA expression in the MPOA of male song sparrow.[22] In contrast, the expression of ERα in the MPOA was elevated in the non-breeding season in male tropical spotted antbirds (*Hylophylax naevioides*).[44] In addition, Gonzales *et al.* have shown that ERβ may act as an inhibitor of ERα transcriptional activity in the developing rat ventromedial nucleus of the hypothalamus.[45] The discrepancies may imply a species-specific effect, while the present data in a wild rodent further add up to the complex roles of ERs in brain physiology. Whatsoever, in this case the varied expression of ERα and ERβ may indicate differential roles in modulating the cyclic changes in hypothalamic function, which need to be further characterized.

In summary, our results demonstrated that AR, ERα, ERβ and P450arom were present in the MPOA of the wild male ground squirrels. Both the protein and mRNA expressions of P450arom and ERβ were higher in the breeding season and pre-hibernation than the non-breeding season. These findings provide novel evidence implicating potentially critical roles of steroid hormones in regulating the hypothalamic function of a wild seasonally-breeding rodent.

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