Steroidogenic enzyme gene expression in the brain of the parthenogenetic whiptail lizard, *Cnemidophorus uniparens*

Brian George Dias\textsuperscript{a}, Sonia Grace Chin\textsuperscript{b}, David Crews\textsuperscript{b,∗}

\textsuperscript{a}Institute for Neuroscience, University of Texas at Austin, Austin, TX 78712, USA
\textsuperscript{b}Section of Integrative Biology, University of Texas at Austin, PAT 30, 1 University Station Stop C 0930, Austin, TX 78712, USA

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\textbf{ABSTRACT}

The steroidogenic enzyme CYP17 is responsible for catalyzing the production of androgenic precursors, while CYP19 converts testosterone to estradiol. De novo neurosteroidogenesis in specific brain regions influences steroid hormone dependent behaviors. In the all-female lizard species *Cnemidophorus uniparens*, individuals alternately display both male-like mounting and female-like receptivity. Mounting is associated with high circulating concentrations of progesterone following ovulation (PostOv), while receptivity is correlated with estrogen preceding it (PreOv). At a neuroanatomical level, the preoptic area (POA) and ventromedial nucleus of the hypothalamus (VMN) are the foci of the male-typical mounting and female-typical receptivity, respectively. In this study, we indirectly test the hypothesis that the whiptail lizard brain is capable of de novo neurosteroidogenesis by cloning fragments of the genes encoding two steroidogenic enzymes, CYP17 and CYP19, and examining their expression patterns in the *C. uniparens* brain. Our data indicate that these genes are expressed in the *C. uniparens* brain, and more importantly in the POA and VMN. Using radioactive in situ hybridization, we measured higher CYP17 mRNA levels in the POA of PostOv lizards compared to receptive PreOv animals; CYP19 mRNA levels in the VMN did not change across the ovarian cycle. To our knowledge, these are the first data suggesting that the reptilian brain is capable of de novo steroidogenesis. This study also supports the idea that non-gonadal sources of steroid hormones locally produced in behaviorally relevant brain loci are central to the mediation of behavioral output.

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\textbf{1. Introduction}

The organization and activation of sexually dimorphic behavioral repertoires by steroid hormones is a central tenet of behavioral neuroendocrinology (Phoenix \textit{et al.}, 1959). For example, male-typical mounting and female-typical receptivity are mediated in part by neural circuits thought to be organized in a sex-typical manner by steroid hormones during development (Schwarz and McCarthy, 2008; Gorski, 2002).

Complementing this organization is the activation of these neural circuits by steroid hormones resulting in sex-typical behaviors in adulthood (Ball and Balthazart, 2004; Baum, 2003). Traditionally, the gonads have been thought to be the primary source of steroid hormones activating sexual behavior. More recently however, the idea of de novo steroid hormone biosynthesis within specific brain nuclei (termed neurosteroids) by the action of steroidogenic enzymes has gained prominence (Baulieu, 1998). A related possibility is that steroid

\textsuperscript{∗} Corresponding author. Fax: +1 512 471 6078.
E-mail address: crews@mail.utexas.edu (D. Crews).

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hormones secreted by the gonads reach the brain via the circulatory system and are then converted to more relevant products in the brain as a result of steroidogenic enzyme activity.

Cholesterol is converted into steroid hormones via several enzyme-catalyzed reactions (Miller, 1988), beginning with its conversion to pregnenolone by the action of cytochrome P450 side-chain cleavage enzyme. Pregnenolone is then converted into either the active progestin, progesterone, by the enzyme 3β-hydroxysteroid dehydrogenase/isomerase or into the androgen, dehydroepiandrosterone (DHEA), by the cytochrome P450 17α-hydroxylase/C17-20lyase enzyme (CYP17) via the 17α-OH pregenenolone intermediate. DHEA can then be converted by 3β-HSD into androstenedione (AE), or AE can be derived from progesterone by the actions of CYP17 via the intermediate 17α-OH progesterone. Androstenedione can be converted into the more active androgen, testosterone (T), by the enzyme 17β-hydroxysteroid dehydrogenase. Finally, T can be converted into the active estrogen, estradiol (E2), by the actions of the enzyme cytochrome P450 aromatase (CYP19). These enzymes are expressed in the nervous system and the neurosteroids resulting from their activity influence adult behavior (Compagnone and Mellon, 2000; Schlinger et al., 2001; King, 2008).

Most studies examining how steroids activate sex-typical behaviors in adulthood do so in gonochoristic animals wherein sex-specific genotypic complements and endocrine histories have already organized behaviorally relevant neuronal circuits in a sex-typical manner (Arnold, 2004; Crews, 2005). An alternative approach to understanding the relationship between steroid hormones and subsequent activation of behavior is to examine organisms which possess a homogeneous genetic background and hormonal milieu (Crews, 2005). The parthenogenetic unisexual whiptail lizard species (Cnemidophorus uniparens) alternates in the expression of both male- and female-typical pseudosexual behavior across the ovarian cycle (Crews and Fitzgerald, 1980). High titers of circulating estrogen in preovulatory (PreOv) animals are correlated with female-like receptivity, while high circulating progesterone levels in postovulatory (PostOv) animals are correlated with male-like mounting. While no androgens are detected in the circulatory system of PostOv individuals (Moore et al., 1985), the nervous system of C. uniparens responds to exogenously administered androgen, with testosterone being a potent activator of male-like mounting in these lizards (Crews et al., 1986; reviewed in Crews, 2005). While the relationship between gonadal steroid hormones and pseudosexual behavior in C. uniparens is well established, several related queries remain. For example, an important question that arises from such hormonal mediation of behavior pertains to whether these hormones are synthesized in the gonad or in the brain. Also, is it possible that local synthesis of androgen in behaviorally relevant brain nuclei resolves the discrepancy between no androgens detected in the circulatory system of mounting postovulatory animals and mounting being elicited by administration of exogenous androgen? A theme common to both these questions implies that de novo neurosteroid synthesis in behaviorally relevant brain nuclei might result in a neurohormonal milieu that is permissive to pseudosexual behavior in C. uniparens. Such a possibility warrants that the nervous system possesses within local circuits the steroidogenic enzymes capable of synthesizing steroid hormones in specific brain nuclei.

Keeping in mind that the preoptic area (POA) and ventromedial nucleus of the hypothalamus (VMN) are involved in male- and female-typical pseudosexual behavior, respectively (Crews, 2005), we hypothesized that higher CYP17 levels and/
or activity in the POA of PostOv lizards might result in androgen synthesis at that locus and be correlated with a male-like behavioral repertoire, while high levels and/or activity of CYP19 in the VMN of PreOv animals might signal estrogen synthesis to facilitate female-like receptivity. As a first step toward testing this possibility, we cloned the C. uniparens CYP17 gene that encodes the enzyme required for biosynthesis of androgenic precursors, and the CYP19 gene encoding the enzyme that catalyzes the conversion of testosterone to estradiol. We then conducted radioactive in situ hybridization on brain tissue obtained from naturally-cycling C. uniparens lizards using probes specific to CYP17 and CYP19.

**Fig. 2**

Alignment of CYP19 cDNA sequences across several vertebrates. Sequence alignment was achieved using ClustalW software. Accession numbers used to compare sequences are shown on the left hand side. Significant identity is observed across species.
CYP19. These are the first data to our knowledge which examine the expression of steroidogenic enzyme mRNA in a reptile brain, and thereby suggest that the reptilian nervous system is capable of de novo neurosteroid biosynthesis. Our results also correlate with the idea that androgen biosynthesis in the POA gates the expression of male-typical mounting, and consequently the switching between sex-typical behavioral repertoires in these lizards.

Fig. 3 – Distribution of cells expressing CYP17 and CYP19 mRNA in selected regions of the C. uniparens brain. The left column denotes neuroanatomical loci, while the middle and right columns depict the localization of CYP17 and CYP19, respectively. Dots only illustrate hybridization signal in that area and make no claim about the quantitative nature of this signal. Scale bar: 0.5 mm.
Fig. 4 – Distribution of cells expressing CYP17 and CYP19 mRNA in selected regions of the C. uniparens brain. The left column denotes neuroanatomical loci, while the middle and right columns depict the localization of CYP17 and CYP19, respectively. Dots only illustrate hybridization signal in that area and make no claim about the quantitative nature of this signal. Scale bar: 0.5 mm.
2. Results

2.1. Cloning of C. uniparens CYP17 and CYP19

A 114 bp fragment of C. uniparens CYP17 (Genbank accession #: EU310876), and a 335 bp fragment of CYP19 (Genbank accession #: EU310875) were cloned from oligo(dT) and random hexamer-primed C. uniparens brain cDNA. The CYP17 fragment shows 78%, 76%, and 82% sequence identity to zebra finch (Taenopygia guttata), snapping turtle (Chelydra serpentina) and human (Homo sapiens) sequences, respectively; while the CYP19 fragment is 81%, 77%, and 74% identical to gecko (Eublepharius macularius), turtle (Trachemys scripta) and mouse (Mus musculus) sequences (Figs. 1, 2).

2.2. Localization of CYP17 and CYP19 mRNA in the brain of C. uniparens

In situ hybridization revealed that both CYP17 and CYP19 had remarkably overlapping expression patterns in the brain (Figs. 3, 4, and Table 1). In the telencephalon, these genes were present in the medial cortex (CxM), dorsal cortex (CxD), and the external nucleus of amygdala (AME). Diencephalic expression was observed in the nucleus periventricularis preopticus (PP), medial preoptic area (MPA), anterior hypothalamus (AH), nucleus dorsolateralis anterior (DL), ventromedial nucleus of the hypothalamus (VMH), periventricular nucleus of hypothalamus (PH), and lentiformis thalami pars plicta (LTP). CYP17 and CYP19 were also found to be expressed in the optic tectum (TECT).

2.3. CYP17 and CYP19 mRNA levels in brain nuclei that are involved in pseudosexual behavior of naturally-cycling C. uniparens

Postovulatory animals have greater levels of CYP17 mRNA in the POA as compared to preovulatory lizards (Two-way ANOVA: Within-subject contrast (Region×Ovarian state): $F_{1,14} = 10.419$, $p < 0.05$; Between-subject (Ovarian state) effect in: $F_{1,14} = 4.731$, $p < 0.05$, Observed power=0.526; Univariate ANOVA with POA mRNA level as dependent variable: $F_{1,14} = 5.837$, $p < 0.05$) (Fig. 5A) ($n = 8$/group). Similar CYP17 mRNA levels were detected in the CxD and VMN across the ovarian cycle. CYP19 mRNA levels (Fig. 5B) in the CxD, POA and VMN were not significantly different between preovulatory and postovulatory lizards. (Two-way ANOVA: Within-subject contrast (Region×Ovarian state): $F_{1,14} = 0.069$, $p > 0.05$; Between-subject (Ovarian state) effect in: $F_{1,14} = 1.355$, $p > 0.05$).

3. Discussion

The cloning of C. uniparens CYP17 and CYP19 from brain tissue, and expression profiles of these genes in the brain suggest that the whiptail lizard brain is capable of synthesizing neurosteroids within specific brain nuclei. Postovulatory

Table 1 - Abbreviations used in brain illustrations (Figs. 3, 4 and 5)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Anterior commissure</td>
</tr>
<tr>
<td>ACC</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>AH</td>
<td>Anterior hypothalamus</td>
</tr>
<tr>
<td>AMC</td>
<td>Central nucleus of amygdala</td>
</tr>
<tr>
<td>AME</td>
<td>External nucleus of amygdala</td>
</tr>
<tr>
<td>CxD</td>
<td>Dorsal cortex</td>
</tr>
<tr>
<td>CXL</td>
<td>Lateral cortex</td>
</tr>
<tr>
<td>CXM</td>
<td>Medial cortex</td>
</tr>
<tr>
<td>DH</td>
<td>Dorsal nucleus of hypothalamus</td>
</tr>
<tr>
<td>DL</td>
<td>Nucleus dorsolateralis anterior</td>
</tr>
<tr>
<td>DM</td>
<td>Nucleus dorsomedialis</td>
</tr>
<tr>
<td>DVR</td>
<td>Dorsal ventricular ridge</td>
</tr>
<tr>
<td>LFB</td>
<td>Lateral forebrain bundle</td>
</tr>
<tr>
<td>LHA</td>
<td>Lateral hypothalamic area</td>
</tr>
<tr>
<td>LPA</td>
<td>Lateral preoptic area</td>
</tr>
<tr>
<td>LTP</td>
<td>Lentiformis thalami pars plicta</td>
</tr>
<tr>
<td>MPA</td>
<td>Medial preoptic area</td>
</tr>
<tr>
<td>NS</td>
<td>Nucleus sphericus</td>
</tr>
<tr>
<td>NSA</td>
<td>Anterior nucleus of septum</td>
</tr>
<tr>
<td>NSL</td>
<td>Lateral nucleus of septum</td>
</tr>
<tr>
<td>NSM</td>
<td>Medial nucleus of septum</td>
</tr>
<tr>
<td>OT</td>
<td>Optic tract</td>
</tr>
<tr>
<td>PC</td>
<td>Posterior commissure</td>
</tr>
<tr>
<td>PH</td>
<td>Periventricular nucleus of hypothalamus</td>
</tr>
<tr>
<td>PP</td>
<td>Nucleus periventricularis preopticus</td>
</tr>
<tr>
<td>SC</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SO</td>
<td>Supraoptic nucleus</td>
</tr>
<tr>
<td>STR</td>
<td>Striatum</td>
</tr>
<tr>
<td>TECT</td>
<td>Optic tectum</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial nucleus of hypothalamus</td>
</tr>
</tbody>
</table>
lizards had higher CYP17 mRNA levels in the POA compared to preovulatory animals, while no changes in CYP19 mRNA levels were detected in the VMN across the ovarian cycle. These data suggest the possibility that androgen biosynthesis within the POA correlates with the activation of male-like mounting, despite the lack of detectable circulating levels of androgen at this time.

Pseudosexual behavior in the unisexual whiptail lizard, *C. uniparens* has proven to be an excellent framework within which to study the activation of sex-typical behavioral repertoires ([Crews, 2005](#)). Of direct relevance to our results are the observations that implantation of dihydrotestosterone and testosterone into the POA and of estrogen into the VMN of the parthenogenetic lizard male- and female-like pseudosexual behavior, respectively ([Wade and Crews, 1991; Kingston and Crews, 1994; Rand and Crews, 1994; Wennstrom et al., 1999](#)). In addition, estrogen implantation into the POA does not elicit mounting, while androgen implantation into the VMN does not facilitate receptivity. These data would suggest a specificity of hormonal action to mediate behavior at distinct brain nuclei, with androgen signaling at the POA mediating male-like mounting and estrogen action at the VMN being critical to female-like receptivity. While exogenously administered androgens robustly elicit male-like mounting in *C. uniparens*, they are undetectable in the circulatory system of postovulatory lizards that mount like males when circulating progesterone levels are high ([Moore et al., 1985](#)). Thus, these animals have retained the sensitivity to androgens but exhibit only a female-like physiology. Several hypotheses might be postulated to explain this discrepancy. For example, progesterone might exert its action via binding to progesterone receptors present in the same cell as androgen receptors, thereby resulting in a convergence of hormonal signaling on neural circuits mediating mounting. An alternative hypothesis tested in this study is that steroid hormones might be synthesized de novo in the brain, and/or precursors such as progesterone from the gonads can be converted into androgen or estradiol at specific brain nuclei. Both these possibilities are predicated on the presence of steroidogenic enzymes in behaviorally relevant brain nuclei.

Several data point to the conclusion that the brain is a site of de novo neurosteroidogenesis. First, genes for the steroidogenic enzymes (mentioned in the Introduction) that are fundamental to de novo neurosteroidogenesis have been cloned and their expression patterns documented in mammalian, avian and fish brains ([Stromstedt and Waterman, 1995; Shen et al., 1994; Saldaňa and Schlenger, 1997; Matsunaga et al., 2001; London et al., 2003; Tomy et al., 2007](#)). Second, these enzymes are catalytically active in the brain and result in functional steroid hormone action at behaviorally relevant nuclei ([Holloway and Clayton, 2001; Balthazart et al., 2004; Cornil et al., 2006](#)). The presence of genes encoding steroidogenic enzymes in the *C. uniparens* brain adds to the cited literature and indicates that neurosteroids can be synthesized at several brain loci in the reptilian brain. The localization of CYP17 in the POA of these lizards emphasizes that androgens could be synthesized in a brain nucleus (POA) involved in male-typical mounting. CYP17 activity and consequently de novo androgen biosynthesis and action in the POA might result in male-typical mounting. Alternatively, CYP17 activity might serve to convert circulating high levels of circulating progesterone in postovulatory animals to testosterone in the POA with the same end result. Both these possibilities address how androgens elicit male-like mounting in these lizards despite not being detectable in the circulatory system following ovulation. Similarly, CYP19 activity and the resulting estradiol biosynthesis and action in the VMN might be related to female-typical receptivity in these lizards.

The whiptail CYP17 and CYP19 genes are expressed in similar spatial profiles as seen in birds and fish in behaviorally relevant diencephalic nuclei of the hypothalamus, in addition to expression in other forebrain and midbrain regions ([Shen et al., 1995; London et al., 2003; Goto-Kazeto et al., 2004](#)). It is important to note that our reported experimental design and analysis does not allow us to distinguish whether CYP17 and CYP19 transcripts are expressed in either neurons or glia or both cell types as has been documented in the case of birds (neurons) ([Cornil et al., 2004](#)) and the midshipman (glia) ([Forlano et al., 2005](#)). The expression patterns of these genes also overlap with that of steroid hormone receptors ([Young et al., 1994; Menuet et al., 2003; London and Schlenger, 2007](#)), thereby suggesting that local neurosteroidogenesis might stimulate receptors within the same brain nuclei to mediate neurosteroid effects. Evidence has accumulated for the role of local preoptic aromatase activity resulting in rapid estrogen biosynthesis being coupled to male sexual behavior in the male quail and rat ([Balthazart et al., 2006; Taziaux et al., 2007](#)). These data taken together illustrate that the stimulation of steroid receptors within brain nuclei by steroid hormones locally produced via the action of steroidogenic enzymes is potentially a conserved mechanism via which the avian, piscian and reptilian nervous systems have evolved to mediate sexually differentiated behavior.

Nervous systems and more specifically brain nuclei within these systems are often sexually differentiated in terms of structure and function. For example, the song system in songbirds is permissive to song production by virtue of being masculinized in males but not in females ([Schlinger, 1998; Arnold, 2000](#)). Using an *in vitro* culture system, recent evidence suggests that more estradiol synthesized de novo in the HVC of male, but not of female zebra finches serves to masculinize the HVC-RA circuitry ([Holloway and Clayton, 2001](#)). Another study postulates that steroidogenic enzymes resulting in estrogen biosynthesis in the brain might play a role in sexual differentiation of the protandrous black pengy fish ([Tomy et al., 2007](#)). Male-like mounting and female-like receptivity expressed by *C. uniparens* are sexually differentiated traits mediated by the POA and VMN, respectively. We have recently published data indicating that the serotonergic system at the POA and the VMN is responsive to the circulating hormonal milieu and serotonergic signaling at the POA serves to gate the expression of male-like mounting in these lizards ([Dias and Crews, 2008](#)). Although the present animal model cannot address the question of the evolution of sex roles as they result from the hybrid union of extant sexual species, if one considers that the first sex was female, and males were derived much later in evolution, it stands to reason that behavior associated with ovulation (i.e., female-like receptivity)
is the ancestral behavioral state and behavior associated with the delivery of sperm (i.e., male-like mounting) is a derived state. This implies that receptivity is a tonically active behavioral repertoire, and that mounting is gated. The lack of any change across the ovarian cycle in CYP19 mRNA levels in the VMN involved in female-like receptivity points toward the idea that the system is primed to be receptive irrespective of hormonal state. The observed increase in CYP17 mRNA levels in the POA of PostOv animals might be accompanied by a corresponding increase in CYP17 expression, activity and testosterone biosynthesis, resulting in an androgen-mediated cascade of events being initiated at the POA thereby permitting male-like mounting. Such an interpretation is consistent with the idea that the POA serves to gate the expression of male-like mounting in several vertebrates (Domínguez and Hull, 2005; Dias and Crews, 2008). We are aware that this speculation is only indirectly corroborated by the data we are reporting in this study, but as mentioned before, these data are a first step toward testing this idea. Future studies would do well to examine protein expression, activity and neurosteroid levels in the POA and VMN of these animals. Of critical importance also would be expression, activity and testosterone biosynthesis, resulting in an androgen-mediated cascade of events being initiated at the POA thereby permitting male-like mounting. Such an interpretation is consistent with the idea that the POA serves to gate the expression of male-like mounting in several vertebrates (Domínguez and Hull, 2005; Dias and Crews, 2008). We are aware that this speculation is only indirectly corroborated by the data we are reporting in this study, but as mentioned before, these data are a first step toward testing this idea. Future studies would do well to examine protein expression, activity and neurosteroid levels in the POA and VMN of these animals. Of critical importance also would be functional studies that inhibit CYP17 action at the POA subsequently inhibiting male-like mounting, and administration of CYP19 (aromatase) inhibitors into the VMN to inhibit female-like receptivity. While our data support the idea that the reptilian brain is capable of de novo steroidogenesis, a more definitive corroboration of this possibility would necessitate the demonstration of the presence of all steroidogenic enzymes that convert cholesterol to estrogen.

In summary, our study suggests that the nervous system of the parthenogenetic whiptail lizard, C. uniparens is capable of de novo neurosteroid biosynthesis, and that such neurosteroidogenesis might serve to explain the hormonal mediation of pseudosexual behavior.

### 4. Experimental procedures

#### 4.1. Animals

Adult, naturally cycling C. uniparens collected from New Mexico and Arizona in the summer of 2007 were used in this experiment. Lizards were housed in a temperature-controlled chamber in sand-filled terrariums and fed crickets on alternating days three times a week, as outlined in Woolley and Crews (2004). All protocols were carried out in accordance with NIH and UT-IACUC animals care and use guidelines.

#### 4.2. Cloning of C. uniparens CYP17 and CYP19 genes

Total RNA was extracted from the C. uniparens brain using the Trizol method (Invitrogen Corp., CA). The total RNA was reverse transcribed using the first strand cDNA synthesis kit (Invitrogen Corp., CA), incorporating oligoT and random hexamers as primers. The resulting cDNA library was used as template in polymerase chain reactions (PCRs) using a degenerate nested primer strategy (see Table 2), with the following conditions: 94 °C for 1 min for denaturation, designated annealing temperature for 1 min, and 72 °C for extension for 35 cycles. A final extension cycle for 10 min at 72 °C was added only in Round 2.

#### 4.3. Naturally-cycling C. uniparens

Postovulatory (PostOv) and preovulatory (PreOv) ovarian states were determined by abdominal palpation. Female-like receptivity was then tested by introducing the experimental animal into the tank of an ovariectomized and testosterone-implanted stimulus animal that had previously shown robust male-like pseudocopulation. In addition, ovarian morphology was noted after sacrificing the experimental animal. All PreOv animals were characterized by the presence of developing follicles and a receptive phenotype, while PostOv animals had corpora lutea and were non-receptive. While the PostOv animals were not tested for male-like mounting behavior, their ovarian morphology was consistent with the exhibition of male-like mounting as demonstrated in the original and subsequent reports (Crews and Fitzgerald, 1980; Moore and Crews, 1986; Dias and Crews, 2008). It is also important to note the mutually exclusive nature of the normal switching between male- and female-like pseudosexual behaviors across the ovarian cycle. PreOv animals are receptive and do not mount, while PostOv animals mount and are not receptive.

#### 4.4. In situ hybridization

In situ hybridization was conducted on every sixth section of the whiptail brain as per Dias and Crews (2008). Briefly, 20 μm fresh frozen coronal sections were generated on a cryostat and thaw mounted on SuperFrost Plus Slides (Erie Scientific, NH). Slides were then fixed in 4% paraformaldehyde, acetylated, and dehydrated prior to storage at –80 °C. Riboprobes specific to the lizard CYP17 and CYP19 genes were transcribed from inserts ligated into the TOPO PCR II vector (Invitrogen Corp., CA) using T7 and SP6 RNA polymerases. All cRNA probes were transcribed using 35S-labeled UTP (PerkinElmer, MA). Slides

<table>
<thead>
<tr>
<th>Gene (PCR Round)</th>
<th>Primer 1</th>
<th>Primer 2</th>
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<td>CYP17 (Round 1)</td>
<td>TGACCTGGGGACAGTHTYGGKNC</td>
<td>CACGGCGCGATTCCXRNARACYT</td>
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<tr>
<td>CYP17 (Round 2)</td>
<td>TGACCTGGGGACAGTHTYGGKNC</td>
<td>CACGGCGCGATTCCXRNARACYT</td>
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<tr>
<td>CYP19 (Round 1)</td>
<td>CCGGATGCTTCATCTCACTNACCATTGNC</td>
<td>CCAGGATGCCCTTTCATNACCATTGNC</td>
</tr>
<tr>
<td>CYP19 (Round 2)</td>
<td>CCGGATGCTTCATCTCACTNACCATTGNC</td>
<td>CCAGGATGCCCTTTCATNACCATTGNC</td>
</tr>
</tbody>
</table>
were incubated for 16–18 h at 55 °C with hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM tris pH 7.4, 1× Denhardt’s solution, 10 mM dithiothreitol, 250 μg/ml yeast tRNA, 10% dextran sulphate, 50 μg/ml herring sperm DNA) and 35S-labeled probe-receptor-specific riboprobes at a concentration of 1×10^6 cpm/150 μl. After hybridization, the tissue was washed in 2× SSC at RT, treated with RNase A (10 μg/ml) at 37 °C for 20 min, followed by stringent washes in decreasing concentrations of SSC with a final wash in 0.25× SSC at RT. Slides were air dried and exposed to BioMAX-MR (Kodak, USA) for 2 weeks. Neither hybridization of RNase pre-treated slides with antisense probe nor sense riboprobe hybridization of experimental slides yielded significant hybridization confirming the specificity of the signal observed with the antisense riboprobes. CYP17 and CYP19 receptor mRNA levels were measured by an experimenter blind to the ovulatory status of the animals using Scion Image (Scion, USA) after performing in situ hybridization. To correct for non-linearity, 14C standards were used for calibration purposes. The use of 14C standards when using 35S labeled probes is routine practice because of a similar emission spectrum of 35S and 14C (Patel et al., 2008; Winzer-Serhan et al., 1999; Vaidya et al., 1997). Optical density measurements using a grid size of 5 × 5 pixels were obtained from both sides of 3–4 individual sections from each animal after the specific regions were outlined. Given that the slides were not coated with emulsion and subsequently processed, we cannot make any statement about the numbers of cells expressing the mRNA of interest.

4.5. Statistical analysis

Statistical analysis was performed using SPPS v12.0 for Windows with the significance set at p < 0.05. For each gene, a two-way ANOVA was conducted using ovarian state (PreOv vs. PostOv) (n = 8/group) as the between-subject variable and mRNA level in the POA, VMN and CxD as the within-subject variable. Only if a significant result (between-subject effect) was obtained in the above ANOVA, data were further decomposed for each region using a univariate ANOVA (independent variable: ovarian state, dependent variable: mRNA in specific region). Given the comparison between only two groups (PreOv vs. PostOv) for the mRNA level of each gene in a specific region, a post-hoc test was not used in the univariate ANOVA.

Acknowledgments

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