Research Report

The distribution of estrogen receptor β mRNA in male and female green anole lizards

Rachel E. Cohen\textsuperscript{a,*,1}, James Roach\textsuperscript{b}, Juli Wade\textsuperscript{a,c,d}

\textsuperscript{a}Department of Zoology, Michigan State University, East Lansing, MI 48824-1101, USA
\textsuperscript{b}Department of Biological Sciences, Florida International University, Miami, FL 33199-2516, USA
\textsuperscript{c}Department of Psychology, Michigan State University, East Lansing, MI 48824-1101, USA
\textsuperscript{d}Department of Neuroscience Program, Michigan State University, East Lansing, MI 48824-1101, USA

\section{Introduction}

Estrogens are steroid hormones that perform critical functions during both development and adulthood. Through their receptors, they mediate a wide range of processes, including reproductive behavior, cardiovascular physiology, neuroprotection, and brain differentiation. These roles are mediated through both genomic and non-genomic mechanisms (reviewed in Vasudevan and Pfaff, 2008). Several types of estrogen receptors (ER) have been described, including ER\textsubscript{α}, ER\textsubscript{β}, and a set of membrane ERs (Micevych and Dominguez, 2009). ER\textsubscript{α} and ER\textsubscript{β} (also known as ESR1 and ESR2, respectively) act at the genomic level, while membrane ERs activate intracellular signaling cascades.

Keywords: Preoptic area, Amygdala, Ventromedial hypothalamus, Anolis carolinensis

Estrogens are critical for a variety of aspects of brain development and adult processes. These steroids act via receptors within specific tissues. Several estrogen receptors (ER) are thought to exist, including ER\textsubscript{α} and ER\textsubscript{β}, which function via classical, genomic mechanisms. These two ERs are found in a variety of species and are critical to diverse functions, including reproductive behaviors. ER\textsubscript{β} was discovered more recently than ER\textsubscript{α}, and very little work has been done on this receptor in reptiles. Currently no data are available on its distribution in the brain in this vertebrate group. Here, we have cloned ER\textsubscript{β} in the green anole lizard, mapped its distribution using in situ hybridization, and quantified expression in three brain areas controlling reproductive behaviors—the preoptic area, ventromedial amygdala (AMy), and ventromedial hypothalamus (VMH). ER\textsubscript{β} was detected in discrete areas throughout the anole brain, with high levels in limbic regions and motor nuclei in the brainstem. Females had a greater density of ER\textsubscript{β} positive cells in the AMy and VMH than males. While the functional consequences of these differences are not clear, they may result in an increased ability to respond to local levels of estradiol. The present work documents that neural ER\textsubscript{β} is distributed similarly in reptilian, rodent and avian species, suggesting that it may perform similar roles. However, more work is necessary to elucidate the function of ER\textsubscript{β} in this group.

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ERβ was first described in rats (Kuiper et al., 1996; Shughre et al., 1996), and has been found in many vertebrate species including humans, Japanese quail, Atlantic croakers, and midshipman fish (Foidart et al., 1999; Hawkins et al., 2005; Mosekmel et al., 1996). Knock-out mice have demonstrated differing roles for ERα and ERβ (Hewitt and Korach, 2003; Rissman et al., 1997). For example, female reproductive behavior in mice is mainly mediated by ERα, with sexual behavior abolished in ERα knockouts, whereas the fertility of ERβ knock-out mice is only slightly impaired (Krege et al., 1998; Ogawa et al. 1998). In male mice, ERα and ERβ knockouts individually have modestly inhibited sexual behaviors, and these behaviors are eliminated when both ERα and ERβ are knocked out (Ogawa et al. 2000; Tetel and Pfaff, 2010). Thus, ERα and ERβ might have many roles in various species and are clearly important for sexual behaviors.

Although it has been characterized in some vertebrate groups, ERβ has not been widely studied in reptiles. For example, the receptor has been documented in the liver of a lizard (Podarcis sicula) and gonads of alligators (Katsu et al., 2004; Verderame and Limatola, 2010), but neural ERβ mRNA has only been examined in the homogenized brain of developing geckos and adult rat snakes (Endo et al., 2008; Katsu et al. 2010). Thus, the anatomical distribution of ERβ in the reptilian brain is unknown. The green anole lizard (Anolis carolinensis) is an excellent model for this work, with much of the anole genome sequenced (Alfoldi et al., 2011). Additionally, the neural anatomy and reproductive behaviors of this species have been well characterized (Greenberg, 1982; Greenberg and Noble, 1944). 17β-Estradiol (E2) production in this species is critical for female receptivity and facilitates the motivation for sexual behavior in males (Latham and Wade, 2010; Winkler and Wade, 1998). Thus, E2, and likely its receptors, are important in producing both male and female sexual behaviors. Although potential sites of action have not been fully evaluated, the binding of 3H-E2 was described more than 30 years ago in this species (Morrell et al., 1979), but only ERα has been mapped (see below).

In many vertebrates, including anoles, the brain areas that are important in the control of male reproductive behaviors include the preoptic area (POA), and amygdala (in green anoles: the ventromedial portion, AMY; Greenberg et al., 1984; Houtsmler et al., 1994; Hull and Dominguez, 2007; Kostarczyk, 1986; Wheeler and Crews, 1978). The ventromedial hypothalamus (VMH) is important in controlling female receptivity in many species, and likely performs a similar function in anoles (Emery and Moss, 1984; Kendrick et al., 1995; La Vaque and Rodgers, 1975). The neural distribution and endocrine regulation of ERα has been investigated in both sexes in green anoles (Beck and Wade, 2009a, 2009b). This receptor is present in the anole POA, AMY, and VMH (Beck and Wade, 2009b). Expression in both sexes is increased in the VMH compared to the other two regions, and is modulated by E2 within this area (Beck and Wade, 2009a). While these data suggest that ERs play a role in the regulation of reproductive behaviors in this species, E2 may also act via ERβ.

The main goal for this experiment was to map the distribution of ERβ in the green anole lizard brain using in situ hybridization with a digoxigenin labeled probe. To do so, we first needed to clone a portion of this gene and develop appropriate probes. We also assessed whether ERβ is likely to play differential roles in male and female sexual behaviors by using stereological procedures to determine whether its expression is sexually dimorphic in regions associated with these functions.

2. Results

2.1. Clone identity and probe specificity

The anole ERβ clone we developed corresponded to bases 187–1331 of the anole ERβ sequence (GenBank XM_003214282; Table 1). The top 50 results from a BLASTx (NCBI) search consisted of ERβ in a diverse set of vertebrates with 75–90% sequence identity. The anole ERβ probe was not similar to anole ERα (GenBank AF095911.2) such that sequence alignment between ERα and our ERβ probe indicated identity of only 34.1% (EMBOSS Alignment Tool). Similarly, our ERβ sequence was a poor match for the green anole androgen receptor (GenBank XM_003227211.1; 41.0%) and progesterone receptor (GenBank XM_003219304.1; 38.0%). In contrast, our ERβ probe was 100% identical to the corresponding bases from the anole ERβ gene. Thus, our probe is specific to ERβ, and under the stringent hybridization conditions used (see Experimental procedures) would not hybridize to these other steroid receptors, or other unrelated sequences.

In situ hybridization using the antisense ERβ probe produced clear cytoplasmic staining, while reactions with the sense probe showed no labeling (Fig. 1). This control indicates that the signal we detected with the antisense probe was due to sequence-specific mRNA binding. Additionally, no labeling was detected in sections where the probes were omitted. This control provides additional confirmation that reagents other than the probe (such as the antibody) do not induce non-specific labeling.

2.2. Overall distribution of ERβ

The distribution and relative intensity of ERβ mRNA expression did not differ qualitatively between the sexes, so the composite data are indicated on a single map (Fig. 2). Representative brain sections, including the three areas where expression of ERβ mRNA was quantified, as well as two brainstem motor nuclei, show high ERβ mRNA labeling (Fig. 3). Cells expressing ERβ were present throughout the brain, with dark labeling (Fig. 1a) in limbic regions and brainstem motor nuclei. Light labeling (Fig. 1b) was also detected in specific areas across the brain.

2.3. Expression in reproductive forebrain areas

Using stereological procedures, we estimated the total number of cells expressing ERβ in the POA, AMY and VMH. We also

Table 1 – Primers used to clone ERβ from the green anole oviduct. Melting temperatures (Tm) are indicated.

<table>
<thead>
<tr>
<th>Sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>55.68</td>
</tr>
<tr>
<td>Reverse</td>
<td>62.66</td>
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determined the density of these cells by dividing the number of cells by the estimated volume within each of the regions.

In the POA, we found no sex difference in the number or density of ERβ expressing cells, or in the volume of the region as defined by ERβ mRNA expression (all t<1.70, p>0.128; Fig. 4a; Table 2).

In the AMY, females had a greater density of ERβ expressing cells than males (t=4.66, p=0.003; Fig. 4b). No other differences were detected on the number of cells expressing ERβ or on the volume of the AMY (all t<2.17, p>0.073; Table 2).

In the VMH, females had a greater density of ERβ expressing cells than males (t=2.39, p=0.049; Fig. 4c). No sex differences were detected in the number of ERβ positive cells, or volume of the VMH as defined by ERβ mRNA expression (all t<1.3, p>0.238; Table 2).

3. Discussion

In the present study, we developed a green anole ERβ clone, described the distribution of this receptor in the brain, and quantified expression in three brain areas controlling reproduction. The results are discussed below in context with data from other species.

3.1 Distribution of ERβ

ERβ mRNA was widely distributed, with relatively high expression in limbic regions and brainstem motoneurones. In particular, the three regions we examined in this study, the POA, AMY, and VMH, all had intense ERβ labeling as did visual motor nuclei and the brainstem region controlling dewlap extension. Thus, ERβ is expressed in limbic regions commonly associated with the motivation and production of sexual behavior (see Introduction), as well as brainstem areas associated with the production of courtship signals (Amb IX/IVm; Wade, 1998). Studies of ERα expression in other species have produced similar results. For example, this receptor is strongly expressed in the POA and portions of the amygdala (avian nucleus taeniaceae) in both rats and Japanese quail (Foidart et al., 1999; Shughrue et al., 1997). ERβ is also present in the POA in male sheep (Hileman et al., 1999) as well as the POA, amygdala, and VMH in Tungara frogs (Chakraborty and Burmeister, 2010). In addition to limbic regions, ERβ is found in the cerebellum of both rats and Japanese quail and the spinal cord of rats (Lakaye et al., 1998; Shughrue et al., 1997). Thus, the pattern of ERβ expression in anoles is largely similar to the distribution of the receptor in other species.

The distribution of ERβ expression in anoles is also consistent with previous work that used autoradiography to demonstrate E2 binding throughout the anole brain (Morrell et al., 1979). No research has been done on membrane ERs in anoles, but research on ERα showed similar expression patterns as ERβ. However, ERα was detected at lower levels in the POA and AMY compared to the VMH (Beck and Wade, 2009b). Autoradiography data suggested that there was high binding of E2 in all three of these regions (Morrell et al., 1979), which, taken with the data on ERβ, suggest that another ER is acting at the POA and AMY. Because we detected high expression of ERβ in these two areas, our data suggest that ERβ may, in fact, be an important site of E2 action in the POA and AMY. Future work should address this hypothesis.

3.2 ERβ in reproductive brain nuclei

We found no sex differences in the total number of ERβ expressing cells in the POA, AMY, or VMH. Additionally, there were no sexual dimorphisms in the volume of these regions, as defined by ERβ expression. In contrast, sex differences in the density of ERβ positive cells were detected, such that this measure was greater in females than males in both the VMH and AMY. Density was calculated by dividing the number of cells expressing ERβ by the volume of the region; neither variable was statistically different between the sexes. Therefore, the sexual dimorphisms we found in the density of ERβ expressing cells reflect the relationship between the number of ERβ positive cells and volume of the regions.

It is possible that the sex difference in ERβ expressing cells we detected stems from sex differences in overall cell density. Previous work from our lab has examined total neuron number in and volume of the POA, AMY, and VMH using similar stereological procedures in Nissl-stained tissue (Beck et al., 2009).
We used those data to calculate the densities of total cells present in the three regions. In contrast, to the data on ER\(\beta\) from the current experiment, the density of cells overall (based on Nissl-staining) was greater in females than males only in the POA (t=2.48, p=0.025). No sex differences existed in the overall density of cells in the AMY or VMH (t<1.99, p>0.070). These data suggest that the sex differences in density of ER\(\beta\) expressing cells in the AMY and VMH are not due to differences in overall cell density, but instead specifically reflect a sexual dimorphism in the density of cells expressing detectable ER\(\beta\). Presumably the increase in females compared to males in these two brain regions reflects an up-regulation of the number of receptor mRNAs per cell. Future work would be needed to determine whether this hypothesis is valid, as that variable cannot be assessed with the labeling technique used in this study. In addition, in situ hybridization does not provide any information on protein availability. Thus, it is possible that sex differences in local receptor availability exist in these regions on at least a couple of levels, and future studies should investigate these issues in more detail.

At present it is unclear what the biological relevance of the density of cells expressing ER\(\beta\) may be. However, assuming that an increase in the density of mRNA containing cells results in a corresponding increase in the number of receptor mRNAs per cell, it is possible that sex differences in local receptor availability exist in these regions on at least a couple of levels, and future studies should investigate these issues in more detail.

Fig. 3 – Representative photomicrographs depicting ER\(\beta\) expression throughout the anole brain including the POA (a), AMY (b), and VMH (c). Two brainstem regions are represented, which include the oculomotor nucleus (d) and the glossopharyngeal portion of the nucleus ambiguus and the ventral motor nucleus of the facial nerve (e). Arrows indicate the boundaries of the brain regions. Males are depicted in all panels. OT = optic tract, 3V = third ventricle, 4V = fourth ventricle. Scale bar = 100 \(\mu\)m (a–d) or 50 \(\mu\)m (e).
in an increase in the availability of functional receptors in a specific area, the potential exists for increased responsiveness to E2 on a local level.

3. Conclusions

This study is the first to examine ER$\beta$ distribution in the reptilian brain. We document a similar pattern as that found in birds and mammals, which suggests that this receptor may perform similar functions across multiple vertebrate taxa. We also found a female-biased sex difference in the density of ER$\beta$ expressing cells in the VMH and AMY. The presence of ER$\beta$ in three limbic nuclei that control reproduction suggests that E2 may act via this receptor in these regions to regulate the display of sexual behaviors. Future work will be necessary to determine the role of ER$\beta$ in the reptilian brain, especially in regard to its specific function(s) in the POA, AMY, and VMH.

4. Experimental procedures

4.1. Animals and tissue processing

Wild-caught adult male and female green anole lizards were purchased from Charles Sullivan (Nashville, TN) during the breeding season (April). They were housed individually in 10-gallon aquaria that contained peat moss, rocks, sticks for climbing, and water dishes. Animals were kept on a 14:10 light/dark cycle and ambient temperatures ranged from 28 °C during the day to 19 °C at night. Full spectrum and heat lamps were provided above each cage to allow basking temperatures of 10 °C above ambient. Relative humidity was maintained at approximately 70%. Cages were misted daily with water and the animals were fed crickets or mealworms dusted with calcium phosphate powder three times a week.

After acclimating to the lab for 2 to 3 weeks, animals were rapidly decapitated. The brains were collected, frozen in methylbutane on dry ice, and kept at −80 °C until processing. Breeding state was confirmed by visual inspection of the reproductive system of each lizard. All females had at least one yolking follicle and males had large, well vascularized testes. Brains were sectioned coronally on a cryostat at 20 μm into four alternate series and thaw-mounted onto SuperFrost Plus (Fisher Scientific; Hampton, NH) slides. They were stored at −80 °C with dessicant until further processing.

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**Table 2 – Estimated mean total number of cells expressing ER$\beta$ in and volume of the POA, AMY, and VMH.** Sample sizes are indicated below. Standard errors are in parentheses. M = male; F = female.

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Number of cells expressing ER$\beta$</th>
<th>Volume ($\times 10^4 \mu m^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POA</td>
<td>M (n=5): 3324 (399)</td>
<td>M: 1493 (152)</td>
</tr>
<tr>
<td></td>
<td>F (n=5): 2417 (356)</td>
<td>F: 1194 (125)</td>
</tr>
<tr>
<td>AMY</td>
<td>M (n=5): 2082 (216)</td>
<td>M: 1317 (963)</td>
</tr>
<tr>
<td></td>
<td>F (n=3): 2465 (186)</td>
<td>F: 1024 (581)</td>
</tr>
<tr>
<td>VMH</td>
<td>M (n=4): 2793 (112)</td>
<td>M: 1867 (150)</td>
</tr>
<tr>
<td></td>
<td>F (n=5): 2799 (189)</td>
<td>F: 1615 (121)</td>
</tr>
</tbody>
</table>

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All procedures adhered to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Michigan State University (protocol # 01/10-003-00).

4.2. Cloning of ERβ

Oviductal mRNA was extracted from two breeding females. Tissue was homogenized in Trizol (Invitrogen Corporation; Carlsbad, CA), and RNA was separated using chloroform. RNA was then isolated using RNase minicolumns (Qiagen Sciences; Valencia, CA) and concentrated with ethanol precipitation. The RNA was reconstituted in DEPC-treated water and stored at −80 °C. It was then converted into cDNA with the SuperScript III Reverse Transcriptase kit (Invitrogen) per manufacturer’s instructions, and stored at −20 °C until use.

Primers were designed for the anole ERβ gene (GenBank: XM_003214282) using the Oligo Analysis Tool program (Eurofins MWG Operon; Huntsville AL; Table 1) and purchased from Invitrogen. PCR reactions included 1 U Platinum TAQ High Fidelity DNA polymerase (Invitrogen), 0.2 mM dNTP mixture, 0.2 μM primer mix, 3 mM MgSO₄, and template cDNA from the oviduct. The PCR reaction went through 40 cycles of 94 °C for 15 s, 50 °C for 30 s, and 68 °C for 1 min. The amplicon was ligated to the vector using the pGEM-T Easy Vector System (Promega) per manufacturer’s instructions.

One Shot TOP10 Chemically Competent E. coli cells (Invitrogen) were transformed with the ligated vectors. The transformed cells were grown overnight on LB agar plates containing 100 μg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). The next day, white colonies were selected and grown overnight in LB broth containing 100 μg/ml of ampicillin, and vector DNA was isolated using Wizard Plus Miniprep kits (Promega). The sequence of the insert was confirmed in both directions on a Perkin Elmer/Applied Biosystems 3100 capillary sequencer and then DNA was isolated using Wizard Plus Maxiprep kits (Promega) and stored at −20 °C.

4.3. In situ hybridization

Sense (SP6) and antisense (T7) probes were transcribed using the Digoxegenin RNA Labeling Kit per manufacturer’s instructions (Roche Diagnostics; Indianapolis, IN). The labeling fractionator from Stereo Investigator (MicroBrightfield, Inc.; Williston, VT) was used to estimate the number of ERβ positive cells in each area (POA: 1000 μm², AMY: 4000 μm², VMH: 8000 μm²), and sampling sites (30×30 μm²) were placed randomly within the defined region. After sampling, estimates of the number of cells and the volume of the region were determined and a Guderson coefficient near 0.1 was confirmed. The density of ERβ expressing cells was calculated by dividing the estimate of the total number by the volume of the region.

4.4. Stereological analysis

Slides were examined under brightfield illumination (as in Cohen and Wade, 2010; Cohen and Wade, 2011). The computerized fractions from Stereo Investigator was used to estimate the number ofERβ positive cells and the volume of the brain region for the POA, AMY and VMH. ERβ positive cells were defined as those cells with distinct dark blue cytoplasmic labeling. Brain areas were determined using reptilian atlases (von Dohna and Nieuwenhuys, 1979; Greenberg, 1982; Babas-Henry and Lohman, 1984) and the region of interest was traced in each section in which it existed. The software placed a grid over each area (POA: 100×100 μm², AMY: 40×40 μm², VMH: 80×80 μm²), and sampling sites (30×30 μm²) were placed randomly within the defined region. After sampling, estimates of the number of cells and the volume of the region were determined and a Guderson coefficient near 0.1 was confirmed. The density of ERβ expressing cells was calculated by dividing the estimate of the total number by the volume of the region.

4.5. Statistical analysis

Each region was analyzed separately using average values from the two sides of the brain for each individual. Two-tailed Student’s t-tests for independent samples were conducted using SPSS to determine the effect of sex on the number and density of ERβ expressing cells in, as well as the volume of the POA, AMY and VMH as defined by ERβ-positive cells. Due to histological artifact, all three areas could not necessarily be analyzed in each individual and final sample sizes are indicated in Fig. 3.

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