DEVELOPMENTAL EXPRESSION OF ESTROGEN RECEPTOR BETA IN THE 
BRAIN OF MICROTUS OCHROGASTER

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DEVELOPMENTAL EXPRESSION OF ESTROGEN RECEPTOR BETA IN THE

BRAIN OF MICROTUS OCHROGASTER

Stephanie Zito

Thesis

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ABSTRACT

There are two primary nuclear estrogen receptors (ER) subtypes, α and β. While there is a large body of research on the role of ERα in regulating social behavior and in expression within the CNS there is significantly less known about ERβ. This is due in part to the fact that the existence of ERα has been known for much longer and in part to the difficulty in visualizing ERβ. Primary antibodies developed for labeling ERβ have had limited success in rats and mice and none have worked in prairie voles (Microtus ochrogaster). Here for the first time we characterize the expression of ERβ-immunoreactivity (IR), using immunocytochemistry, in the brains of prairie voles. ERβ-IR was compared in juveniles, 21-days of age, and in adult males and females, 60 days of age. Results indicate several major findings. First, unlike ERα expression, ERβ expression is not sexually dimorphic, with males and females expressing similar patterns in the brain. Second, ERβ express an adult pattern by day 21 as there were no age dependent effects on distribution. Finally, ERβ in the prairie vole may not be as wide spread as reported in rats and mice. High levels of ERβ-IR were observed in several regions/nuclei within the medial pre-optic area, ventrolateral pre-optic nuclei and in the hypothalamus, especially the
paraventricular and supraoptic nuclei. The visualization of ERβ in prairie voles is important as the socially monogamous prairie vole functions as a human relevant model system for studying the expression of social behavior and social deficit disorders and ER is known to play a role in the expression of social behavior. Future studies will now be able to determine the effect of treatments on the expression and/or development of ERβ in this highly social species.
ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Primary antibodies developed for labeling ERβ have had limited success in rats and mice and none have worked in prairie voles (Microtus ochrogaster). We have recently developed a primary antibody and here for the first time, visualize and describe ERβ-IR in the brains of prairie vole. Being able to visualize ERβ is critical for studying and understanding the effects of estrogen acting through this major ER subtype.

Statement of the Problem

This study aims to determine the expression of ERβ in the prairie vole (Microtus ochrogaster). The research was designed to determine not only the expression, but also if expression is sexually dimorphic or age-dependent, by evaluating ERβ expression in male and female prairie voles at two ages, day 21 (D21) and day 60 (D60).
Importance of the Study

Determining the location and role of ERβ in regulating prairie vole social behavior is important for several reasons. First, ERβ has, at least in part, been characterized in the mouse and rat, but never in the prairie vole. Second, future studies will be able to determine the effect of treatments on the expression and/or development of ERβ. Third, more information regarding ERβ will provide direct evidence for physiological significance in the interaction between estrogen and other neuropeptides. Lastly, the socially monogamous prairie vole functions as a human relevant model system for studying the expression of social behavior and social deficit disorders and ER is known to major a role in the expression of social behavior.
CHAPTER II

BACKGROUND OF THE STUDY

Historical Survey of Previous Work in the Area

While frequently thought of as a “reproductive” hormone estrogen is not only critical to male and female reproduction it also plays an important role during development, in maintenance and physiology of the body, and is critical for the development and expression of behavior. It affects male and female reproductive tissue and non-reproductive systems such as the cardiovascular, skeletal, and central nervous systems (CNS). The effects of estrogen are regulated by the actions of estrogen via the two primary nuclear ER subtypes, α (alpha) and β (beta). ERα was first isolated in 1986 (Greene et al., 1986), which lead to accelerated studies on the function of ERα regulating social behavior and in expression within the CNS. The second receptor type, ERβ, was first identified in 1996 (Kuiper et al., 1996). However, although reported more than 10 years ago, there is significantly less known about ERβ. This is due in large to difficulties in “visualizing” ERβ.

Trouble with the specificity of ERβ antibodies makes it difficult to visualize the location of ERβ. The most extensive information regarding the distribution of
ERβ in the CNS comes from the rat model. ERβ is expressed in the brain and spinal cord. The receptors tend to have overlapping expression with a few exceptions where either ERα or ERβ is not reported, or one of the receptors dominates in expression (Weiser et al., 2007). Unlike ERα, which is sexually dimorphic in a few nuclei in rats and more so in prairie voles, reports of ERβ mRNA indicate that ERβ distribution is the same in both intact male and female rats (Laflamme et al. 1998).

Brain regions such as the bed nucleus of the stria terminalis (BST), medial and cortical amygdaloid nuclei, and preoptic area (POA) express both forms of ER mRNA (Laflamme et al., 1998). ERα protein mostly dominates in the medial preoptic area (MPOA), arcuate nucleus and the ventromedial nuclei of the hypothalamus (Chung et al., 2007). While only ERβ mRNA is found in the olfactory bulb, supraoptic (SON), paraventricular (PVN), suprachiasmatic (SCN), and tuberal hypothalamic nuclei (Chung et al., 2007).

While Laflamme et al. (1998) and Chung et al. (2007) both worked on the expression in the rat brain, Mitra et al. (2003) were able to first describe ERβ-ir in the adult ovariectomized mouse brain. They reported localization of ERβ staining in the nuclei of cells in the olfactory bulb, cerebral cortex, septum, POA, BST, amygdala, paraventricular hypothalamic nucleus, thalamus, ventral tegmental area, substantia nigra, dorsal raphe, locus coeruleus, and cerebellum. Although both receptors were expressed in similar distribution, Mitra et al. (2003) showed that ERα protein was the predominant subtype in the mouse hippocampus, POA, and most of the hypothalamus.
Whereas, ERα has been shown to play a definite role in social and sexual behavior (Mazzucco et al., 2008), ERβ may have more of an accessory role. Some of the more recent research has been able to show the correlation of ERβ expression with social behaviors such as learning and memory, anxiety, social interaction/aggression, and reproductive behavior (Bodo and Rissman, 2006).

The Two Receptors

ERα and ERβ are not true isoforms because the receptors are coded by genes on different chromosomes (Enmark et al., 1997). However, sequence identity between them is 97% for the DNA-binding domain and 60% for the ligand-binding domain (Bodo and Rissman, 2006). The ERβ ligand-binding domain is smaller than ERα and differs by two amino acids (Agatonovic-Kustrin et al., 2008). In general, ligands selective for ERβ binding are smaller and more polar than those that are selective for ERα. ERα has a greater binding affinity to estradiol due to its bulky size (Li et al., 2008) however; there are also regions of ERα that will selectively bind estradiol only in the presence of ERβ (Lui et al., 2007). Therefore, the mode of interaction of estrogenic compounds depends on the receptor subtype; for example, tamoxifen is an agonist and antagonist of ERα and a pure antagonist of ERβ (Mazzucco et al., 2008).

ER are unique from other sex hormone receptors, because they do not require a specific steroidal structure for binding (Agatonovic-Kustrin et al., 2008), therefore some man-made and naturally occurring substances directly activate these receptors and can interfere with their normal functions. When activated the
ERs mediate many nuclear and non-nuclear events critical to the functioning of various tissues by interacting with specific DNA sequences called estrogen response elements (ERE). ERβ has been shown to be a weaker transcription factor than ERα in the presence of estradiol (Li et al. 2008). The amino-terminus of ERα is a multi-functional domain critical for regulating the potential of the receptor, whereas this same region in ERβ is responsible for impairing the receptor’s interaction with ERE.

The idea that these receptors function through different signaling pathways has been suggested in understanding the mechanisms in which they activate estrogen-regulated gene expression, for ERα and ERβ have the ability to regulate the expression of both the same and distinct genes. Lui et al. (2008) attempted to distinguish whether these differences were due to DNA-binding properties of the receptors or interactions with co-regulators. They found that ERβ tends to activate ERE-binding closer to the transcription start site (TSS) and recruit ERα binding.

Recent studies have found variations in ERα and ERβ mRNA splicing in rodents and humans (Price et al., 2000). Five different splice variants have been described for ERβ thus far, with the original form being designated ERβ1, each arising from alternative splicing of the eight exons (Weiser et al., 2007). ERβ2 has an in-frame insertion between exons five and six, resulting in an additional 18 amino acids in the ligand-binding domain. It has been shown to bind estradiol with approximately a 10-fold lower affinity than the ERβ1 variant (Chung et al., 2007. ERβ1δ3 has a deletion of exon three, which codes in the DNA-binding
domain. ERβ2δ3 has the in-frame insertion between exons five and six, as well as the deletion of exon three. Finally, ERβ1δ4 is missing exon four and appears to not bind to estrogen.

**ERβ and Other Neuropeptides**

Several neurotransmitter-containing neurons have been found to co-express ERβ, which suggest a role for estrogen in mediating the effects of other neurotransmitters. Previous studies have shown the ERβ1 protein colocalized with 88% of GnRH neurons in ovariectomized female rats (Hrabovszky et al., 2001) and ERβ2 colocalized with 71% of GnRH neurons (Chung et al., 2007). Chung et al. (2007) have suggested that this colocalization with GnRH may be for control of transcriptional activity, where ERβ1 acts in a dominant-negative fashion through heterodimerization. ERβ2 may also be involved in regulating the serotonergic system, as evidenced by abundant levels of ERβ2-IR in areas of the adult female rat which express high levels of 5HT1A receptor mRNA (Chung et al., 2007). These findings suggest that ERβ2 might play an important role in regulating sleep, mood, anxiety, cognitive function and have been linked to the serotonergic system.

Albertson et al. (2008) reported Gonadotropin Releasing Hormone-I Receptor (GnRHR)-labeled neurons in the mouse and sheep brain were also immunoreactive for ERβ, suggesting that steroids are likely to affect GnRHR expression. Simonian and Herbison (1997) reported double labeling of ERβ in the medial and lateral parvocellular divisions of the PVN of approximately 35% of
oxytocin neurons. However, very little colocalization (<5%) was seen with vasopressin neurons in the PVN (Laflamme et al., 1998). This may be responsible for the effects of estrogen in mediating the suckling-induced oxytocin, but not vasopressin, neuronal and gene expression in the hypothalamus of lactating mothers (Laflamme et al., 1998). These observations provide evidence for physiological significance in the interaction between estrogen and other neuropeptides through this ER subtype.

Estradiol has been shown to increase progesterone receptor (PR) gene expression within specific regions of the brain. Gonzales et al. (2008) have previously reported that estradiol increases PR levels in the medial preoptic nucleus (MPN) 50-fold, but does not significantly affect PR levels in the ventromedial nucleus of the hypothalamus (VMN). When comparing this data with the localization of ER\(\alpha\) and ER\(\beta\), Gonzales et al. (2008) suggested that ER\(\beta\) is inhibiting ER\(\alpha\), thus reducing the sensitivity of the VMN to estradiol.

Estrogen and Behavior

ER\(\alpha\) and ER\(\beta\) have distinct biological functions, which is supported by their specific expression patterns and the different phenotypes observed in ER\(\alpha\) and ER\(\beta\) knockout mice (Heldring et al., 2007). Kudwa et al. (2003) showed that ER\(\alpha\) is critical for the expression of female receptivity in mice. The ability to produce offspring and the lordosis reflex in ER\(\alpha\) knockout (KO) mice were completely absent, indicating that ER\(\alpha\) is necessary for female sexual behavior. Whereas,
ERα has been shown to play a definite role in sexual behavior, ERβ may have more of an accessory role.

Some of the more recent research has been able to show the correlation of ERβ expression with social behaviors such as learning and memory, anxiety, social interaction/aggression, and reproductive behavior. In the hippocampus estradiol affects synaptic plasticity resulting in increased performance in learning and memory tasks (Walf et al., 2008; Liu et al., 2008). ERβ has been reported in the hippocampus of mice suggesting a high likelihood that at least some of the effects of estradiol are being mediated through ERβ (Walf et al., 2008). Walf et al. (2008) believe that these effects of estradiol may be a combination of rapid, membrane-mediated action and intracellular ERs in the hippocampus. Laflamme et al. (1998) suggest that ERβ may play a role in how an individual reacts to stress by modulating such responses as blood pressure and other autonomic-related functions, through the selective transcription of the CRFergic population in the PVN.

Estrogen can have either an anxiolytic or anxiogenic activity, which could be mediated by the different receptor subtype. Lund et al. (2005) treated rats with the selective ERβ agonist diarylpropionitrile (DPN) and saw a decrease in anxiety-related behaviors. Whereas the ERα agonist propyl-pyrazole-triol (PPT) increased anxiogenic behaviors suggesting that ERβ may contribute to mediating anxiolytic, while ERα anxiogenic activity. In some of the same research on anxiety, Krezel et al. (2001) also reported a disruption in the GABAergic system of the amygdala. Hughes et al. (2008) used the ERβ agonist, WAY-2000070, and
described a reduced immobility time in the mouse tail suspension test indicating antidepressant-like effects of ERβ.

Finally, Kudwa et al. (2005) found that estrogen acts through ERβ to defeminize the developing male brain and prevent female-like behaviors. When tested on male mice lacking functional ERβ, treatment with estrogen and progesterone produced female patterns of sexual receptivity. In contrast, the down-regulation of hormone receptors by their ligands has been reported for several steroid hormones and Al-Bader et al. (2008) suggested estradiol down-regulation of its receptors may play a significant role in the developing brain while the fetus is exposed to very high levels of circulating maternal estrogen. Consequently, the fetal brain may be less responsive to estrogens during late pregnancy therefore defeminization must occur earlier in development.

Model Organism

The relationship between development, early social experiences, and subsequent behavior has been examined in many mammalian species including humans. Social monogamy is a rare behavioral trait that is expressed in humans, and between 3 to 5% of all mammalian species (Klieman 1977). It is characterized by the formation of pair bonds between mates (Getz et al., 1981) and reduced sexual dimorphism. These interactions generally result in biparental care.

In typical human relationships the father and mother have the most interactions with their offspring, however siblings and more distant relatives often
play a part in social interactions. Prairie voles are a good model for this type of behavior because they are socially monogamous, and therefore have been used as a human-relevant rodent model system for studying the expression and regulation of social behavior.

Early social interactions can play a critical role in the development and subsequent expression of social behavior. It has been hypothesized that early social experience can have an organizational effect on ER and thereby alter the subsequent expression of social behavior (for a review see Cushing and Kramer, 2005). The neonatal period is when a significant amount of social interaction occurs. In social species, these interactions can include the mother, father, siblings and even distant relatives. This is also the time when significant development is occurring. By describing the expression of ERβ in the prairie vole, we hope to gain a better understanding of the developmental effects of estrogen and how social interactions can alter this effect.
CHAPTER III

DESCRIPTION OF THE WORK OF THE WRITER

Materials and Methods

Experimental animals were obtained from the breeding colony housed in the Biological Resource Center at The University of Akron. Prairie voles were obtained from a stock that originated in Urbana, Illinois. All animals were maintained on a 14/10-hour light/dark cycle in a temperature and humidity controlled environment. High fiber rabbit chow and purified water were provided \textit{ad libitum}. All procedures were approved and in accordance with the Institutional Animal Care and Use Committee of the University of Akron.

Tissue Collection

The location of ER$\beta$ was determined in D21 and D60 prairie voles, animals were deeply anesthetized with a mixture of Ketamine/Xylazine, brains were collected and the location of ER$\beta$-IR determined in males and females (n= 8, per sex and age). Brains were fixed in 4% buffered paraformaldehyde and 5% acrolein using a modified spinning immersion technique described in Kramer et
al. (2005). Fixed brains were stored in 25% sucrose at 4°C until they were sectioned at 30 µm on a freezing sliding microtome. Sections were stored at -20°C in cryoprotectant until they were processed for immunocytochemistry (ICC) staining for ERβ.

Immunocytochemistry

Free-floating sections were rinsed in a potassium phosphate buffer solution (KPBS) and then incubated for 20 minutes in 10% sodium borohydride. Next sections were incubated with rabbit ERβ antibody formed from the last 18 amino acids from the N terminus of ERβ (Proteintech Group Chicago, Illinois) at a dilution of 1:7000 in 0.05M KPBS-0.4% Triton X-100 for 48 hours at 4°C. The specificity of the antibody was confirmed by preabsorption with the synthetic peptide used to produce the ERβ antibody, using ten times the concentration of antibody. The preabsorption was visualized by using a nickel-diaminobenzine chromogen solution (0.25 g nickel sulfate, 10 mg DAB, 8.3 µl 3% Hydrogen Peroxide in 10 ml 0.175 M sodium acetate) instead of the glucose oxidase-diaminobenzine chromogen solution. Verification was also determined by following the above protocol without incubation in the primary antibody.

After incubation in the primary antibody, the tissue was rinsed in KPBS and then incubated for 1 hour at room temperature (RT) in biotinylated goat anti-rabbit IgG (1:600 dilution in 0.4% Triton X-100)(Vector Burlingame, CA). Sections were then incubated in an avidinbiotin peroxidase complex (Vectastain ABC Kit-Elite pk-6100 standard; Vector, Burlingame, CA; 4.5 µl A and 4.5 µl B per 1 ml
solution) for 1 hour. Sections were rinsed in KPBS, followed by Tris buffer (pH 7.2). Finally, ERβ was visualized by using a glucose oxidase-diaminobenzidine chromogen solution (0.004 g ammonium chloride, 0.02 g β-D-glucose, 10 mg DAB, 1.5 µl glucose oxidase in 10 ml Tris buffer.) Sections were mounted onto super-frost plus glass slides, dried, dehydrated, in ascending ethanol solutions (70%, 90…ECT), cleared in Histoclear, and cover slipped using Histomount. Image analysis with Image Pro Software Version 6.2 on Olympus model #BX51 was used to determine the average number of cells expressing ERβ in each brain region. The Rat Brain by George Paxinos and Charles Watson (6th edition) was used to determine the brain regions where staining was present.

Western Blot

Antibody specificity to ERβ was additionally confirmed by western blot analysis under the following conditions. Protein extracts from a single brain were prepared in SDS lysis buffer (0.5% SDS, 50mM Tris, 1mM DTT, pH 8) as a 10% homogenate. Total protein was collected by centrifugation at 16,000-x g after precipitation with 10 volumes acetone and quantified using a standard Bradford assay (ThermoFisher Scientific, Waltham, MA). Electrophoresis of 30 µg protein was completed on 12.5% denaturing gels followed by semidry transfer to polyvinylidene fluoride membranes. Immobilized proteins were incubated 1 hr in TTBS blocking buffer (100 mM Tris, 150mM NaCl, 0.1% Tween-20, 5% nonfat dry milk, pH 7.6). The primary antibody, rabbit anti-vole ERβ anti-sera, was diluted 1:2500 in blocking buffer and incubated at 1 hr at room temperature.
After two washes in TBS (100 mM Tris, 150 mM NaCl, pH 7.6) the secondary antibody, a monoclonal mouse anti-rabbit HRP conjugate (Sigma), was diluted 1:25,000 in blocking buffer and incubated at room temperature 2.5hrs. Membranes were then rinsed in TBS and reactive proteins were detected using a Kodak Gel Logic 2200 Imaging System upon addition of Super Signal West Pico chemiluminescent substrate (ThermoFisher Scientific, Waltham, MA). This study did not evaluate differences in splice variant expression.

Statistical Analysis

Using the Jump® program, the differences in the average number of cells expressing ERβ-IR were determined using a nested two-way ANOVA for age and sex in all brain areas with strong to moderate staining. Statistical tests were conducted separately for each brain region. Results were considered significant at $P > 0.05$.

Results

In the present study we documented the localization of ERβ in the brain of the prairie vole. This work represents the first description of ERβ distribution in the prairie vole brain. Data from select brain regions will be highlighted below, and our findings will be compared with the previously reported data in the rat and mouse.
Specificity of the Antibody

The results from both the western blot and immunocytochemistry confirmed the specificity of our ERβ primary antibody. Western blotting identified a band migrating at approximately 56-70-kDa (Fig. 1.1, lane 2). The band falls within the known range for ERβ reported in other species, which ranges around 70-kDa (Mitra et al. 2003) in the mouse brain and to 56-60-kDa band reported by Chung et al. (2007).

Use of this antibody for immunocytochemistry showed strong nuclear staining of cells containing ERβ and this labeling was eliminated by prior incubation with the peptide (Fig. 1.2). Therefore, this antibody was determined to be suitable for immunocytochemical studies.

The specificity of the antibody in prairie vole tissue is demonstrated in Fig 1.3. The majority of the staining was detected specifically within cell nuclei, as shown in a representative section of the periventricular hypothalamic nucleus (Fig. 1.3A). Importantly, no staining was present when tissue was incubated with antibody that had been preabsorbed with the peptide (Fig 1.2).

ERβ Localization in the Prairie Vole Brain

Table 1.1 is a comprehensive list of the specific brain regions with ERβ-IR expression.
Figure 1.1 Western blot analysis. Lane 1 is the ladder. Lane 2 is ERβ, size range between 56-70-kDa.
Figure 1.2 Immunocytochemistry: Preabsorption with peptide. Immunocytochemical staining of ERβ cells preincubated with peptide followed by antibody (A) or incubated in antibody (B).
Figure 1.3 ERβ-IR in prairie voles (*Microtus ochrogaster*).
Figure 1.3 ERβ-IR in prairie voles (*Microtus ochrogaster*). (continued)
Figure 1.3 ER\(\beta\)-IR in prairie voles (*Microtus ochrogaster*). (continued) A, paraventricular hypothalamic nucleus (PVN) in female D21 (B), periventricular hypothalamic nucleus (Pe) and supraoptic nucleus (SON) in female D21 (C), SON and PVN in female D60 (D), ventromedial preoptic nucleus (VMPO), anteroventral periventricular nucleus (AVPe), and median preoptic nucleus (MnPO) in female D21 (E), bed nucleus of the stria terminalis (BST) in male D60 (F), supraoptic nucleus retrochiasmatic part (SOR), retrochiasmatic area lateral part (RChL), and dorsomedial hypothalamic nucleus dorsal part (DMD) in female D60 (G), lateral preoptic area (LPO) in male D21 (H), lamina terminalis (LTer) in male D21 (I), septohypothalamic nucleus (SHy) in male D21 (J), sublenticular extended amygdala central part (EAC) in male D21 (K), sublenticular extended amygdala medial part (EAM) in female D60 (L), suprachiasmatic nucleus ventromedial part (SChVM) in male D60 (M), medial amygdaloid nucleus ant dorsal (MeAD), supraventricular zone of the hypothalamus (SPa), and supraoptic decussation (SOX) in female D60 (N), ventrolateral hypothalamic nucleus (VLH) in male D60 (O), VMPO in female D60 (P), PVN in female D60 (Q), PVN in male D21 (R), PVN in male D60 (S), representation of nuclear staining of ER\(\beta\)
Table 1.1 ERβ-IR in prairie voles (*Microtus ochrogaster*)

<table>
<thead>
<tr>
<th>Area</th>
<th>21 Days</th>
<th>60 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>anteroventral periventricular nucleus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>arcuate hypothalamic nucleus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>bed nucleus stria terminalis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lateral division, intermediate part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial division, anterior part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial division, anteromedial part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial division, ventral part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>dorsomedial hypothalamic nucleus, dorsal part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>lateral preoptic area</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>lemina terminalis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial amygdaloid nucleus, ant dorsal</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial preoptic area</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>medial preoptic nucleus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lateral part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>median preoptic nucleus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>paraventricular hypothalamic nucleus</strong></td>
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<td></td>
</tr>
<tr>
<td>anterior part</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>medial part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ventral part</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>peduncular part of lateral hypothalamus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>periventricular hypothalamic nucleus</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>retrochiasmatic area, lateral part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>septohypothalamic nucleus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>sublenticular extended amygdala</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>central part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>medial part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>suprachiasmatic nucleus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ventromedial part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>supraoptic decussation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>supraoptic nucleus</strong></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>retrochiasmatic part</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>supraventricular zone of the hypothalamus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ventrolateral hypothalamic nucleus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ventromedial preoptic nucleus</td>
<td>+</td>
<td>+</td>
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</table>
There was no significant difference in the pattern of expression across age or sex in any of the areas where ERβ-IR was localized. The brain region found to exhibit the greatest concentration of intense ERβ-IR included the anterior part of the paraventricular hypothalamic nucleus. Strong to moderate nuclear ERβ-IR was observed in cells of the ventral part of the paraventricular hypothalamic nucleus, the periventricular hypothalamic nucleus, and the SON.

Cells exhibiting moderate to weak ERβ-IR were observed in the anteroventral periventricular nucleus, arcuate hypothalamic nucleus, various regions of the bed nucleus stria terminalis, dorsal part of the dorsomedial hypothalamic nucleus, lateral preoptic area, lemina terminalis, anterior dorsal medial amygdaloid nucleus, MPOA, medial and lateral parts of the medial preoptic nucleus, median preoptic nucleus, lateral part of the retrochiasmatic area, septohypothalamic nucleus, central and medial parts of the sublenticular extended amygdala, SCN including the ventral part, supraoptic decussation, retrochiasmatic part of the SON, supraventricular zone of the hypothalamus, ventrolateral hypothalamic nucleus, and the ventromedial preoptic nucleus.

Overall, the expression patterns of ERβ were similar between D21 and D60 prairie vole brains. Of the major areas where ERβ expression was present, there were no significant differences across sex or age in the supraoptic nucleus \([F_{(3,27)}=23.10, \text{ P n.s.}]\), paraventricular hypothalamic nucleus medial part \([F_{(3,21)}=6.70, \text{ P n.s.}]\), anterior part \([F_{(3,26)}=403.89, \text{ P n.s.}]\), ventral part \([F_{(3,26)}=55.81, \text{ P n.s.}]\), or periventricular hypothalamic nucleus \([F_{(3,26)}=15.42, \text{ P n.s.}]\).
CHAPTER IV

SUMMARY

Discussion

In the present study I document the localization of the ERβ subtype in the brain of the prairie vole. The results from this study indicated several things. First, the pattern of expression of ERβ was the same in both males and females. This finding differs from ERα in prairie voles, where the expression is highly sexually dimorphic with males expressing less ERα than females (Cushing et al., 2004). This finding is consistent with reports in the rat and mouse. Second, there was no age effect on the expression of ERβ, suggesting that adult pattern is established by the time of weaning. Finally, the results indicate that there may be significant differences in expression of ERβ in prairie voles compared with other species. The results of visualization of ERβ suggest that ERβ expression is more limited in prairie voles than in other rodent species. While prairie voles expressed ERβ in most of the regions/nuclei of the brain that have been reported in rats and mice, there were a number of areas where ERβ was absent in prairie voles.
The novel ERβ peptide antibody selectively recognizes ERβ in prairie tissue, as determined by western blot analysis and immunocytochemistry in prairie vole brain sections. The apparent size of the approximately 70-kDa band detected in rodent brain and human testicular extracts appears to be similar to the 56-70-kDa band seen in prairie vole brain tissue. Although up to five splice variants of ERβ mRNA have been reported in rodents and humans (Price et al., 2000), this study did not evaluate differences in splice variant expression.

Estrogen has been shown to have differing effects in males and females. Unlike ERα, which is sexually dimorphic in a few nuclei in rats (Cushing and Wynne-Edwards, 2006) and more so in prairie voles. Reports of ERβ mRNA indicate that ERβ distribution is the same in both intact male and female rats (Laflamme et al. 1998). Similarities in male and female ERβ distribution are supported in the prairie vole, which supports the idea that ERα plays the key role in the sexually dimorphic effects of estrogen. These results also suggest the possibility that the effects of ERβ in terms of regulating fear and anxiety may be the same in both adult males and females.

Differences in the distribution of ERα between early postnatal and adult have also been reported in the prairie vole (Yamamoto et al., 2006). This suggests a differential role for estrogen acting through ERα throughout development. Whereas, significant differences in ERβ-IR were not observed in the age groups chosen for this study, it should not be assumed that expression patterns are not likely to vary at earlier stages in developing brain tissue that has
not yet been examined. These data should be considered to represent ERβ distribution specifically in the adult intact prairie vole brain.

A comparison of ERβ-IR in the brain of the prairie vole, mouse, and rat provides insight into the role this receptor subtype may play in prairie vole brain function and social behavior. Our data for ERβ expression in the prairie vole brain are generally in agreement with previous reports describing the distribution of ERβ mRNA in the mouse (Mitra et al., 2003) and rat brain (Chung et al., 2007; Laflamme et al., 1998). For instance, as in the rat and mouse, cells immunoreactive for ERβ were found in the preoptic areas, bed nucleus of the stria terminalis, paraventricular hypothalamic nucleus, arcuate nucleus, amygdala, suprachiasmatic nucleus, and supraoptic nucleus. ERβ-IR cells were also found in the periventricular hypothalamic nucleus, lamina terminalis, septohypothalamic nucleus, supraoptic decussation, supraventricular zone of the hypothalamus, and ventrolateral hypothalamic nucleus.

Notable exceptions include the amygdaloid region, where expression of ERβ mRNA has been reported throughout this region in the rat but this study reports expression in only the extended amygdala, cerebral cortex, and the tuberal hypothalamic region. These areas may express levels of mRNA that are not proportional to the translated protein. Other regions such as the olfactory bulb, septum, thalamus, ventral tegmental area, substantia nigra, dorsal raphe, locus coeruleus, and cerebellum were not examined in this study, so correlations to previous works are not possible.
ERβ has been shown to play a role in social behaviors such as learning and memory, anxiety, social interaction/aggression, and reproductive behavior. ERβ has been reported in the hippocampus of mice where it may affect synaptic plasticity and result in increased performance in learning and memory tasks (Walf et al., 2008). While, ERβ was not expressed in the hippocampus in the prairie vole, the comparison may serve as an important role in understanding the processes of learning and memory.

It has been suggested that ERβ may play a role in how an individual reacts to stress by modulating such responses as blood pressure and other autonomic-related functions, through the selective transcription of the CRFergic gene population in the PVN (Laflamme et al., 1998). Further research in the role ERβ plays in regulating stress response especially in the realm of social interactions may be accessible through further studies of the distribution in prairie voles. Also, Lund et al. (2005) reported a decrease in anxiogenic behaviors with treatment of the ERβ agonist suggesting that ERβ may contribute to mediating anxiolytic. Use of agonist and antagonists in the prairie vole may contribute to a better understanding of ERβ in contributing to anxiogenic or anxiolytic behaviors.

While previous research in rat and mouse models have examined colocalization of ERβ mRNA with GnRH, serotonin, vasopressin, and oxytocin containing neurons (Chung et al., 2007; Laflamme et al., 1998; Simonian and Herbison, 1997). It may be interesting to determine whether colocalization of ERβ in the prairie vole brain shows similar results. This information could also help to determine the role ERβ plays in regulating brain function and social behavior.
This work represents the first description of ERβ distribution specifically in the prairie vole brain. In all brain regions with ERβ-IR, it was detectable in the cell nucleus. Although the distribution patterns of the receptor are not significantly different across sex and age, localization of ERβ distribution in the prairie vole brain provides insight into the role this receptor subtype may play in prairie vole brain function and the regulation of social behaviors.


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APPENDIX

Abbreviations Key

BST – Bed nucleus of the stria terminalis
MPN – Medial preoptic nucleus
MPOA – Medial preoptic area
POA – Preoptic area
PVN – Paraventricular hypothalamic nucleus
SCN – Suprachiasmatic nucleus
SON – Supraoptic nucleus
VMN – Ventromedial nucleus of the hypothalamus