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Differential Effects of Estrogen Receptor alpha Suppression by Antisense Oligodeoxynucleotides in the Medial Preoptic Area and the Medial Amygdala on Male Rat Mating Behavior

Jacquelyn Carrie Paisley

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Differential effects of estrogen receptor alpha suppression by antisense oligodeoxynucleotides in the medial preoptic area and the medial amygdala on male rat mating behavior

by

Jacquelyn Carrie Paisley

Under the Direction of Andrew N. Clancy, PhD

Abstract

Male rat copulation is mediated by estrogen-sensitive neurons in the medial preoptic area (MPO) and medial amygdala (MEA); however, the mechanisms through which estradiol (E₂) acts are not fully understood. We hypothesized that E₂ acts through estrogen receptor α (ERα) in the MPO and MEA to promote male mating behavior. Antisense oligodeoxynucleotides (AS-ODN) complementary to ERα mRNA were bilaterally infused via minipumps into either brain area to block the synthesis of ERα, which we predicted would reduce mating. Western blot analysis and immunocytochemistry revealed a knockdown of ERα in each brain region; however, compared to saline controls, males receiving AS-ODN to the MPO showed significant reductions in all components of mating, whereas males receiving AS-ODN to the MEA continued to mate normally. These results suggest that E₂ acts differently in these brain regions to express sexual behavior and that ERα in the MPO, but not in the MEA, promotes mating.

Index Words: Estrogen, Estrogen receptor, Antisense oligodeoxynucleotides, Mating, Medial preoptic area, Medial amygdala
DIFFERENTIAL EFFECTS OF ESTROGEN RECEPTOR ALPHA SUPPRESSION BY ANTISENSE OLIGODEOXYNUCLEOTIDES IN THE MEDIAL PREOPTIC AREA AND THE MEDIAL AMYGDALA ON MALE RAT MATING BEHAVIOR

by

JACQUELYN CARRIE PAISLEY

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the College of Arts and Sciences

Georgia State University

2007
DIFFERENTIAL EFFECTS OF ESTROGEN RECEPTOR ALPHA SUPPRESSION BY ANTISENSE OLIGODEOXYNUCLEOTIDES IN THE MEDIAL PREOPTIC AREA AND THE MEDIAL AMYGDALA ON MALE RAT MATING BEHAVIOR

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JACQUELYN CARRIE PAISLEY

Committee Chair: Andrew N. Clancy
Committee: Laura L. Carruth
Sarah L. Pallas

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2007
DEDICATION

Thanks to so many who have shared this experience with me. I couldn’t have done it without your encouragement and support.

Poppy, thanks for always believing in me.

Mom, I love you! Your love of learning led me down this path. I will never forget word games during bath time. Thank you.

Katt and Sarebear, thanks for all your love and encouragement. You are the stars in my life, and I hope you are as proud of me as I am of you both.

GG, you taught me everything I know and become a cherished friend along the way. You are the one who truly understands what we went through.

Jim, you’ve stuck with me all the way. I guess the never ending story has finally ended. Or, maybe not…

The universe is full of magical things, patiently waiting for our wits to grow sharper.

~ Eden Phillpotts
ACKNOWLEDGEMENTS

At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.

~ Albert Schweitzer

I want to thank my committee members, Drs. Andrew Clancy, Laura Carruth, and Sarah Pallas for their wisdom, knowledgeable input, and patience in the completion of this master’s thesis. At times when my “light” seemed to go out, you pushed me forward. Thank you for your support and never wavering faith in me and our work.
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<tbody>
<tr>
<td>a-p</td>
<td>anterior-posterior</td>
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<tr>
<td>AF-2</td>
<td>activation function 2</td>
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<tr>
<td>AF-1</td>
<td>activation function 1</td>
</tr>
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<td>AP-1</td>
<td>activator protein 1</td>
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<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ArKO</td>
<td>aromatase knockout</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>antisense oligodeoxynucleotides</td>
</tr>
<tr>
<td>BSA-E₂</td>
<td>estrogen conjugated bovine serum albumin</td>
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<tr>
<td>cm</td>
<td>centimeters</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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<td>dorsal-ventral</td>
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<tr>
<td>E₂</td>
<td>estradiol</td>
</tr>
<tr>
<td>EF</td>
<td>ejaculation frequency</td>
</tr>
<tr>
<td>EL</td>
<td>ejaculation latency</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
</tr>
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<td>βERKO</td>
<td>estrogen receptor beta knockout</td>
</tr>
<tr>
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<tr>
<td>αβERKO</td>
<td>estrogen receptor alpha and beta knockout</td>
</tr>
<tr>
<td>EST</td>
<td>eastern standard time</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid</td>
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<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>IF</td>
<td>intromission frequency</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<td>-ir</td>
<td>immunoreactivity</td>
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<td>kilograms</td>
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<td>LBD</td>
<td>ligand binding domain</td>
</tr>
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<td>lmp</td>
<td>liters per minute</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>medial amygdala</td>
</tr>
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<td>MF</td>
<td>mount latency</td>
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<tr>
<td>µg</td>
<td>micrograms</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>MIS</td>
<td>missense oligodeoxynucleotide sequence</td>
</tr>
<tr>
<td>µl</td>
<td>microliters</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>m-l</td>
<td>medial-lateral</td>
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<td>ML</td>
<td>mount latency</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
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<td>MPO</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>N</td>
<td>number</td>
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<tr>
<td>NDM</td>
<td>nonfat dry milk</td>
</tr>
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<td>NDS</td>
<td>normal donkey serum</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>P</td>
<td>progesterone</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PEI</td>
<td>post ejaculatory interval</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAL</td>
<td>saline</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCRAM</td>
<td>scrambled oligodeoxynucleotide sequence</td>
</tr>
<tr>
<td>T</td>
<td>testosterone</td>
</tr>
<tr>
<td>TTBS</td>
<td>tris-buffered saline with tween-20</td>
</tr>
<tr>
<td>VMH</td>
<td>ventromedial hypothalamus</td>
</tr>
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<td>WT</td>
<td>wild type</td>
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INTRODUCTION

The central nervous system controls mating behavior by integrating both peripheral and central stimuli. Sexual chemosensory, visual, auditory and somatosensory cues are received by the brain to promote expression of copulatory behavior, a process that is facilitated by gonadal steroids [Meisel and Sachs, 1994]. Testosterone (T) is released into circulation from the male testes, and their removal results in decreased sexual behavior [Beach and Holz-Tucker, 1949; Davidson, 1966]. Exogenous T, however, can restore mating behavior in long-term castrates or maintain mating if treatment is started at the time of castration [Davidson, 1966; Christensen and Clemens, 1974].

In males, testicular T is irreversibly and extensively converted into several biologically active metabolites, including dihydrotestosterone (DHT) via 5α-reductase [Massa et al., 1972] and estradiol (E2) via aromatase [Naftolin et al., 1975], in both the periphery and the brain. Hormone manipulation studies have shown that these T metabolites, and T itself, contribute to the expression of mating behavior. Studies showed that, unless dispensed at pharmacological doses, neither exogenous DHT [Feder, 1971; McGinnis and Dreifuss, 1989] nor E2 [Davidson, 1969; Södersten, 1973] administered alone effectively restores mating behavior in castrated male rats. However, administered together at physiological doses, DHT and E2 can fully restore or maintain mating behavior [Baum and Vreeburg, 1973; Larsson et al., 1973; Feder et al., 1974], as do physiological doses of T, suggesting that both androgenic and estrogenic metabolites of T promote male sexual behavior. Significant reductions in mating behavior were observed in castrated, T-maintained male rats when the conversion of T to E2 was blocked by the administration of Fadrozole, a non-steroidal aromatase inhibitor [Vagell and McGinnis, 1997], either systemically [Bonsall et al., 1992] or locally into either the medial preoptic area (MPO)
[Clancy et al., 1995] or the medial amygdala (MEA) [Huddleston et al., 2006]. Additionally, these behavioral deficits were partially reversed by systemic E$_2$ administration [Bonsall et al., 1992] or local E$_2$ implants into either the MPO [Clancy et al., 2000] or the MEA [Huddleston et al., 2003]. These studies suggest that the estrogenic metabolites of T contribute significantly to the normal expression of male copulatory behavior.

Convergent evidence from behavioral changes following lesioning [Larsson and Heimer, 1964; Harris and Sachs, 1975], electrophysiological stimulation [Malsbury, 1971; van Dis and Larsson, 1971], and hormone manipulation [Davidson, 1966a, 1966b; Davis and Barfield, 1979] studies suggest that a neuronal circuit composed of interconnected steroid-sensitive neurons forms the substrate that underlies male mating behavior and that key brain regions in this network include, but are not limited to, the MPO and the MEA. Comparisons of Fos immunoreactivity (-ir) in the brains of mated males versus those of unmated controls revealed increased levels of Fos-ir in several brain regions, including the MPO and the MEA [Baum and Everitt, 1992; Coolen et al., 1996], indicating that these areas were active during mating. Further studies revealed that neurons expressing mating-induced Fos-ir in the MPO and the MEA also contained either androgen receptors (AR), estrogen receptors (ER) or both AR and ER, suggesting that these brain regions are steroid sensitive [Greco et al., 1998].

Although it is likely that E$_2$-sensitive neurons of the MPO and the MEA contribute to the expression of male rat sexual behavior, less is known about how E$_2$ acts at the cellular level in either of these brain areas. Both regions express ER$_{\alpha}$ and ER$_{\beta}$ [Shughrue and Merchenthaler, 2001; Greco et al., 2003]; and, whereas knockout of ER can attenuate mating in male mice [Ogawa et al., 1997, 2000; Wersinger et al., 1997], a single ER subtype may have special behavioral significance in any given brain region. Therefore, we sought to selectively remove
ERα from either the MPO or the MEA, hypothesizing that E₂ acts through ERα in these two brain regions to promote mating. To accomplish this, we infused either the MPO or the MEA with antisense oligodeoxynucleotides (AS-ODN) complementary to the translation start site of ERα mRNA.

The central dogma of molecular biology suggests that DNA is transcribed into RNA which is then translated into proteins. AS-ODN are nucleic acid sequences designed to interact with mRNA transcripts according to Watson-Crick pairing rules, thus inhibiting their translations into proteins [Morris and Lucion, 1995; Landgraf, 1996]. In principle, any known gene sequence can be targeted, and its gene product intercepted, resulting in the down regulation of the protein product and, ultimately, a change in target cell function. For example, ERα AS-ODN injected directly into the ventromedial hypothalamus (VMH) of neonates protected against the androgenizing effects of T in the developing female rat brain [McCarthy et al., 1993]; and, when injected into the right lateral ventricle of female rats, ERα AS-ODN resulted in decreased ERα-ir in the VMH and significantly reduced lordosis behavior [Walf et al., 2007]. Most in vivo studies have administered AS-ODN by acute injection into either the ventricles or specific sites in the brain, but very few studies have infused AS-ODN in a chronic manner into specific brain regions and into solid tissue, which was the approach employed in this study. We chronically infused AS-ODN complementary to ERα mRNA into either the MPO or the MEA to test the hypothesis that ERα is the form of ER through which E₂ acts in each of these brain regions for the display of mating behavior. We predicted that AS-ODN infusion in the MPO or the MEA would block the local expression of ERα and reduce male rat copulatory behavior.
EXPERIMENTAL PROCEDURES

Animals

Male and female Sprague-Dawley rats, at least 65 days of age, were obtained from Charles River Laboratories. Animals were housed in polycarbonate cages, 22 x 44 x 18 cm, on a 14:10 hour reverse light:dark cycle (lights off at 0930 hours EST) with free access to food and water. Males were housed two per cage until the day of surgery and were single housed thereafter. Females were housed two per cage throughout the study. All maintenance and surgical procedures were in accordance with institutional regulations and with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, revised 1985).

Stimulus female rats were anesthetized by placing them in a chamber with an atmosphere of 5% isoflurane gas and an oxygen exchange rate of 1000 liters per minute (lpm) before transferring them to a nosecone dispensing 2% gas at 300 lpm. Each female was ovariectomized and implanted subcutaneously (s.c.) with a 6 mm Silastic capsule filled with crystalline E₂. All females were allowed at least seven days to recover before the inception of behavioral testing. On test days, females received 1.0 mg progesterone (P) s.c. (1 mg P/0.2 ml sesame oil) 4-6 hours (h) before being paired with males.

Male rats were screened for sexual behavior in four weekly 30 minute (min) mating tests to determine their suitability for inclusion in the study, and groups of proven maters were matched based on total ejaculation frequency. These males were assigned to one of four groups: 1. MPO AS-ODN (N = 6), 2. MPO SAL (N = 8), 3. MEA AS-ODN (N = 6) or 4. MEA SAL (N = 7).

At the time of surgery, males were anesthetized using isoflurane gas in the same manner as the females. Twenty-eight gauge stainless steel, ethylene oxide sterilized cannulae were
stereotaxically implanted bilaterally into either the MPO (level skull coordinates: a-p = -0.5 mm, m-l = ± 0.75 mm, d-v = -9.0 mm) or the MEA (level skull coordinates: a-p = -3.2 mm, m-l = ± 3.5 mm, d-v = -9.2 mm) [Swanson, 1998]. Each cannula was connected via Tygon microbore tubing to an osmotic minipump (Alzet, model 2004) implanted s.c. in the scapular region. Minipumps infused either AS-ODN dissolved in physiological saline (AS-ODN groups) or physiological saline as a vehicle control (SAL groups) at the rate of 0.25 µl/hr (6 µl/day/cannula) for 28 days. After a 5-7 day surgical recovery period, weekly 30 min sexual behavior tests resumed for a period of 8 weeks. Minipumps were replaced under anesthesia following the fourth postoperative test. Upon removal at week four and at the end of the study, the minipumps were examined to assure there were no pump failures; none occurred.

**Antisense Oligodeoxynucleotide Preparation**

Experimental animals were infused via minipumps with a 25-mer ODN sequence (5’-GTGAAGGGTCATGTCATGTCATG-3’) antisense to the translation start codon for rat ERα [Koike, 1987]. Lyophilized ODN (6290 µg, Oligos Etc. Inc., Wilsonville, OR) was re-suspended in 7.548 ml sterile physiological saline and stored in aliquots at -80°C until use. These concentrated aliquots were further diluted before use to a working concentration of 1 µg AS-ODN/12 µl saline, which was used to fill the pumps for the experimental animals (vehicle controls received minipumps containing saline only). Before implantation, minipumps were incubated in sterile saline solution for 48 h at 37°C for priming.
**Oligodeoxynucleotide Controls**

Following behavioral screening, additional groups of matched males were formed for infusion of an ODN control. In these groups, the MPO or MEA was infused with either a scrambled nucleotide sequence (SCRAM1: 5’ – CTCTACACATCGATCGTGACTG – 3’, MPO N=5, MEA N=5; SCRAM2: 5’ – CTAGGTCTAGCTGTCCACACGTGAG – 3’, MPO N=7) or a missense nucleotide sequence (MIS: 5’ – GTGTAGGGTTATGTCATTGTAAGT – 3’, MPO N=9). These altered sequences were not complementary to ER mRNA or any other known mRNA (GenBank nucleotide-nucleotide similarity BLAST search). In contrast to the AS-ODN infused groups, a majority of the animals in these altered sequence groups were found to display brain morphological or behavioral abnormalities which resulted in death or required humane euthanasia. Therefore, further attempts to develop this type of control were abandoned for ethical reasons.

**Male Sexual Behavior**

*Behavior Testing Protocol*

During preoperative sexual screening and postoperative mating tests, each male was paired with a sexually receptive female for 30 min in a 22 x 44 x 50 cm testing arena under dim red light. Tests began about 3 h after lights off. The following behaviors were recorded by observers blind to the experimental status of the animals: mount frequency (MF): the number of mounts without penile penetration; intromission frequency (IF): the number of mounts with penile penetration; and ejaculation frequency (EF): the number of ejaculations. We also measured mount latency (ML): time from the beginning of the test until the first mount or intromission occurred or a default of 1800 sec if no mounts or intromissions occurred;
ejaculation latency (EL): time from the first mount or intromission until the first ejaculation occurred or a default of 1800 sec if no ejaculations occurred; and the post-ejaculatory interval (PEI): time between the first ejaculation and the next mount or intromission or a default of 1800 sec if no ejaculation occurred.

**Tissue Collection**

*Estrogen Receptor Immunocytochemistry and Cannula Placement*

When the testing period was concluded, half of the males in each group were euthanized with an overdose of sodium pentobarbital (150 mg/kg i.p.) and perfused transcardially for 5 min with physiological saline followed by a minimum of 300 ml of a fixative containing 2% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (PB) solution (pH 7.4). Skulls were then partially opened and immersed overnight in the same perfusion fixative. Brains were removed from skulls the next day and stored in 30% sucrose in 0.1 M PB for approximately 48 h. Frozen coronal brain sections (40 μm) through the diencephalon were collected into 0.1 M PB. One set of sections from each male was mounted on clean gel-albumin coated slides and stained with Toluine blue to verify cannula placement. The remaining sections were stored in cryoprotectant (30% ethylene glycol, 20% glycerol, 50% 0.2 M PB, pH 7.4) at 4°C until used for immunocytochemistry (ICC).

*Western Blot Analysis*

The remaining males in each group were euthanized with an overdose of sodium pentobarbital (150 mg/kg i.p.), and brains were quickly removed from the skulls and placed on an ice cold glass plate. Two coronal razorblade cuts were made at the rostral and caudal
boundaries of the hypothalamus, anterior to the optic chiasm and posterior to the mamillary bodies, respectively, and the poles were discarded. The slab containing the hypothalamus was divided coronally into two parts, a rostral piece containing the MPO and a caudal piece containing the MEA. Bilateral parasaggital cuts at the medial edges of the left and right olfactory tubercules were made in the rostral slab containing the MPO, and the lateral edges of the slab were discarded. Next, a horizontal cut was made ventral to the anterior commissure, and the dorsal piece was discarded; a sagittal midline cut through the third ventricle divided the MPO into left and right halves. In the caudal slab containing the MEA, a horizontal cut was made at the dorsal margin of the internal capsule, and the dorsal piece was discarded. Next, bilateral parasaggital cuts were made at the medial edges of the left and right internal capsule, and the medial piece was discarded leaving two lateral tissue samples containing the left and right MEA. Upon dissection, each sample was transferred immediately to an eppendorf tube and flash frozen in a methanol/dry ice bath. All samples were stored at -80°C until used for protein isolation.

**Estrogen Receptor Immunocytochemistry**

Following a series of washes in 0.1 M PB to remove the cryoprotectant, coronal sections from animals in the four groups were processed concurrently with the same aliquots of antibodies and immunoreagents. Sections were incubated with agitation, first, in a blocking solution containing 5% normal donkey serum (NDS) in 0.1 M PB (pH = 7.4) for 1 h at room temperature (RT) and, second, with the 1D5 anti-ER antibody (Zymed Laboratories, San Francisco, CA, catalog # 18-7149) for 48 h at 4°C. The 1D5 anti-ER antibody, a mouse monoclonal antibody whose epitope is located in the N-terminal domain (A/B region) of ER [a
Saati et al., 1993] and has been found to be specific for ERα [Tomanek et al., 1997; Nishihara et al., 2000], was used at a concentration of 0.75 µg/ml in a solution consisting of 5% NDS in 0.1 M PB. After three 5 min washes in 0.1 M PB, sections were then incubated for 2 h RT with a donkey anti-mouse biotinylated secondary antibody (Jackson Laboratories, Bar Harbor, ME, code # 715-065-151, lot # 48672) at a dilution of 1:200 in 0.1 M PB plus 3% NDS. Sections were then washed in three 5 min changes of 0.1 M PB and incubated for 1 h RT in an avidin-biotinylated peroxidase complex (Vector Labs, Burlingame, CA, Vectastain Elite Standard ABC kit, catalog # PK-6100, A and B reagents prepared at one-tenth the manufacture’s recommended concentration). Following three additional 5 min washes in 0.1 M PB, the sections were incubated for 20 min RT in a solution containing 0.2 mg/ml diaminobenzidine (Sigma-Aldrich, St. Louis, MO, catalog # D-5637, lot # 29H1060) plus 0.1 µl/ml 30% H2O2 (Sigma-Aldrich, St. Louis, MO, catalog # H-1009, lot # 014K3646) dissolved in 0.1 M PB.

**Western Blot Analysis**

**Protein Isolation**

Within each experimental group (MPO AS-ODN (N=2), MPO SAL (N=2), MEA AS-ODN (N=2), and MEA SAL (N=2)), tissue samples from the left side of the brain were pooled and homogenized in 1ml Hepes buffer solution (5 mM Hepes, .3 mM EDTA) with a protease inhibitor cocktail additive (Sigma-Aldrich, St. Louis, MO, catalog #P8340). The homogenates were centrifuged for 25 min at 4°C at 18,200 x g. The supernatant was immediately removed and stored at -80°C until use. The protein concentration for each sample was later determined by a bicinchoninic acid assay (Pierce, Rockford, IL, catalog #23227).
Western Blot

Ten µg of protein from each sample were run on a 10% Tris-HCl gel (Bio-Rad, Hercules, CA) and transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, catalog #162-0180). The membrane was blocked in 5% nonfat dry milk (NDM) in Tween-Tris buffered saline (TTBS) for 4 h and incubated overnight at 4°C in 1D5 (1:500 in 2% NDM in TTBS). Following a series of washes in TTBS, the membrane was incubated at room temperature for 1 h in a horseradish peroxidase linked anti-mouse secondary antibody (1:2000 in 2% NDM in TTBS; Cell Signaling Technology Inc, Danvers, MA, catalog #7076) and was visualized using LumiGLO Chemiluminescent Substrate (Upstate, Lake Placid, NY, catalog #20-212) on Kodak Biomax light film (Sigma-Aldrich, St. Louis, MO, catalog #Z370371-50EA, Kodak #178 8207).

Quantification of Western Blot

Film from the western blot was scanned directly, and the relative optical density (OD) was measured microscopically using Bioquant software (BQ-TCW98, version 3.50.6 MT, R&M Biometrics, Nashville, TN). Fields in the center of each band (750µm x 550µm; 281,805 pixels) were measured under identical conditions of illumination, thresholding on dark pixels (R=92.0; G=94.0; B=86.0) in the darkest field which yielded an optical density value for each band in the western blot.

Statistics

Between-group behavioral data were analyzed by repeated measures (group by trial) analysis of variance, followed by post hoc comparisons using the Tukey honestly significant differences test at a probability level of 0.05, whereas within-group comparisons of preoperative
and postoperative changes in behavior were analyzed by paired t-tests [Kirk, 1968]. The Mann-
Whitney U [Siegel, 1956] test was used to analyze ICC data. Two-tailed probabilities are
reported except if explicitly stated otherwise.
RESULTS

Male Sexual Behavior

Highly significant group differences emerged during the postoperative period in which animals that had been infused into the MPO with AS-ODN virtually ceased mating, whereas the animals in the other 3 groups exhibited robust mating, as indicated by all behavioral indices (FIGURE 1). Specifically, during postoperative trials, the 4 groups differed significantly in mount frequency (group main effect: $F_{3,23} = 10.20, p < 0.001$, trial main effect $F_{7,161} = 2.13, p < 0.043$), intromission frequency (group main effect: $F_{3,23} = 15.47, p < 0.001$), ejaculation frequency (group main effect: $F_{3,23} = 6.48, p < 0.003$), mount latency (group main effect: $F_{3,23} = 6.92, p < 0.002$, trial main effect $F_{7,161} = 2.75, p < 0.01$), ejaculation latency (group main effect: $F_{3,23} = 8.23, p < 0.001$) and the post-ejaculatory interval (group main effect: $F_{3,23} = 13.48, p < 0.001$). Prior to surgery, however, the animals in all 4 groups had mated robustly and the groups were statistically indistinguishable on intromission frequency, ejaculation frequency and the post-ejaculatory interval, although some modest but significant differences were noted on mount frequency (group x trial interaction: $F_{9,69} = 2.07, p < 0.044$), mount latency (group x trial interaction: $F_{9,69} = 2.49, p < 0.016$) and ejaculation latency (group x trial interaction: $F_{9,69} = 2.02, p < 0.049$). Thus, group differences manifested during the postoperative trials.

In a follow-up analysis, because post hoc pairwise comparisons indicated that MPO SAL, MEA AS-ODN and MEA SAL groups were statistically indistinguishable on all indices of copulatory behavior during all postoperative trials, these 3 groups were pooled; and the sexual performance of this combined control group was subsequently compared with that of animals in the MPO AS-ODN group. Infusion of the MPO with AS-ODN profoundly suppressed mating behavior in comparison to that of the combined control group. Specifically, during postoperative
FIGURE 1: MALE SEXUAL BEHAVIOR. Preoperative and postoperative mating behaviors (mean ± standard error) of all groups. Males infused with saline to the MPO or MEA mated normally, as did males infused with AS-ODN to the MEA; whereas, males receiving AS-ODN to the MPO virtually ceased mating. Blackened symbols indicate the trials where post hoc group comparisons revealed that the MPO AS-ODN group differed significantly from the other groups (p < 0.05, two-tailed). Asterisks (*) indicate behaviors in which the within group comparison of preoperative trials and terminal postoperative performance differed significantly (see text).
trials, the 2 groups differed significantly in mount frequency (group main effect: $F_{1,25} = 32.60, p < 0.001$; trial main effect: $F_{7,175} = 2.11, p < 0.040$), intromission frequency (group main effect: $F_{1,25} = 46.94, p < 0.001$; trial main effect: $F_{7,175} = 2.07, p < 0.049$; group x trial interaction: $F_{7,175} = 3.02, p < 0.005$), ejaculation frequency (group main effect: $F_{1,25} = 12.85, p < 0.002$), mount latency (group main effect: $F_{1,25} = 15.21, p < 0.001$; trial main effect: $F_{7,175} = 2.73, p < 0.010$; group x trial interaction: $F_{7,175} = 2.38, p < 0.024$), ejaculation latency (group main effect: $F_{1,25} = 21.31, p < 0.001$) and the post-ejaculatory interval (group main effect: $F_{1,25} = 39.45, p < 0.001$; trial main effect: $F_{7,175} = 2.72, p < 0.011$). Subsequently, post hoc group comparisons indicated significant differences ($p < 0.05$) in mount frequency on trials 2, 3, 4, 5, 7 and 8, intromission frequency on all trials but the first, ejaculation frequency on trials 3 and 5, mount latency on trials 3, 5, 6 and 7, ejaculation latency on trials 3, 5 and 6 and the post-ejaculatory interval on all trials but the first.

In the MPO AS-ODN group, for each dependent variable, there was a significant decline in sexual performance between the preoperative and postoperative periods. Specifically, for mount frequency, comparisons of preoperative performance (averaged over 4 preoperative trials) with postoperative performance (averaged over the last 4 postoperative trials, which represents terminal postoperative performance) indicated that mounting declined significantly in frequency during the postoperative period ($t = 3.18, df = 5, p < 0.025$). Similarly, comparisons of the same sets of trials indicated that intromission frequency declined significantly during the postoperative period ($t = 5.37, df = 5, p < 0.003$), as did ejaculation frequency ($t = 2.68, df = 5, p < 0.044$), whereas mount latency ($t = 2.79, df = 5, p < 0.038$), ejaculation latency ($t = 3.10, df = 5, p < 0.027$) and the post-ejaculatory interval ($t = 3.57, df = 5, p < 0.016$) increased significantly. In marked contrast to the deterioration of mating that occurred in the MPO AS-ODN group, there
were no significant changes on any behavioral measure between preoperative and postoperative
periods in the MPO SAL (infused with saline to the MPO), and MEA AS-ODN (infused with
AS-ODN to the MEA) groups. In the MEA SAL group (infused with saline to the MEA), except
that intromission frequency was significantly lower postoperatively than preoperatively (t =
4.15, df = 6, p < 0.006), the animals in this group also exhibited robust mating both before and
after surgery, and no other behavioral measures differed significantly between the preoperative
and postoperative periods.

*Estrogen Receptor Immunocytochemistry*

Chronic AS-ODN infusion inhibited ERα expression as assessed by ERα ICC. Infusion
of the MPO (FIGURE 2) or MEA (FIGURE 3) with AS-ODN suppressed ERα-ir neuronal
labeling in the MPO or MEA, respectively; whereas, saline infusion of either brain area was
accompanied by appreciable ERα-ir labeling. Specifically, significantly fewer ERα-ir neurons
were detected in the MPO of rats in the MPO AS-ODN group (mean ± standard error: 4.88 ±
3.18) than were found in the MPO of rats in the MPO SAL group (20.88 ± 2.83), U = 1, n1 = 5,
n2 = 3, p < 0.036, 1-tail. Moreover, significantly fewer ERα-ir neurons were detected in the
MEA of rats in the MEA AS-ODN group (0.00 ± 0.00) than were found in the MEA of rats in
the MEA SAL group (12.79 ± 5.70), U = 0, n1 = 4, n2 = 4, p < 0.028. No significant differences
in ERα-ir neuronal counts were detected in the arcuate nucleus of either the MPO AS-ODN and
MPO SAL groups (33.99 ± 10.46 and 29.17 ± 12.50, respectively, p > 0.05) or the MEA AS-
ODN and MEA SAL groups (30.54 ± 9.32 and 58.33 ± 11.14, respectively, p > 0.05).
FIGURE 2: ESTROGEN RECEPTOR IMMUNOCYTOCHEMISTRY IN THE MPO. Sections through the MPO (top row) and ARC (bottom row) from males in the MPO AS-ODN (left column) and MPO SAL (right column) groups were processed concurrently with the 1D5 anti-ERα antibody. ERα-ir labeling was reduced significantly (see text) in the MPO of males in the MPO AS-ODN group as compared to the MPO of males in the MPO SAL group. Abundant ERα-ir labeling occurred in the ARC in both cases suggesting that infusion of the AS-ODN blocked expression of ERα in the MPO only.
FIGURE 3: ESTROGEN RECEPTOR IMMUNOCYTOCHEMISTRY IN THE MEA. Sections through the MEA (top row) and ARC (bottom row) from males in the MEA AS-ODN (left column) and MEA SAL (right column) groups were processed concurrently with the 1D5 anti-ERα antibody. ERα-ir labeling was reduced significantly (see text) in the MEA of males in the MEA AS-ODN group as compared to the MEA of males in the MEA SAL group. Abundant ERα-ir labeling occurred in the ARC in both cases suggesting that infusion of the AS-ODN blocked expression of ERα in the MEA only.
Western Blot Analysis

The 1D5 antibody recognized protein bands at approximately 67 kDa, which is the previously reported size for full length rat ERα [Koike et al., 1987; Furlow et al., 1990]. The band corresponding to the MPO SAL group was darkest suggesting an appreciable concentration of ERα in the MPO; whereas, the band associated with the MPO AS-ODN group was substantially lighter. Quantification of the western blot indicated an approximate 4-fold knockdown of ERα by AS-ODN infusion of the MPO (FIGURE 4). Although overall ERα expression was lower in the MEA as compared to the MPO, similar results were observed in the MEA in which quantification of the western blot revealed an approximate 6-fold knockdown of ERα by AS-ODN infusion of the MEA (FIGURE 4). These results are consistent with those obtained from ERα ICC.

Cannula Placements

Locations of cannula placements to the MPO are indicated in FIGURE 5 and those to the MEA in FIGURE 6 (see Tissue Collection in Experimental Procedures). Cannula tips were positioned in or near the MPO or MEA in all groups of animals.
FIGURE 4: RELATIVE OPTICAL DENSITY OF WESTERN BLOT ANALYSIS. Relative optical density was measured for each band found at 67kDa in the western blot, and expressed as a percentage based on the total pixel count in the field evaluated. The bands corresponding to the SAL groups in both the MPO and the MEA contained the highest percentage of dark pixels (50.24% and 22.14%, respectively); whereas, bands corresponding to the AS-ODN groups in both the MPO and the MEA contained fewer dark pixels (13.18% and 3.79%, respectively) suggesting that AS-ODN infusion blocked expression of ERα in both brain regions. Fields sampled within the bands are pictured below the bars for each group.
FIGURE 5: CANNULA PLACEMENTS TO THE MPO. Cannula tips were positioned in or near the MPO in the MPO AS-ODN and MPO SAL groups.
FIGURE 6: CANNULA PLACEMENTS TO THE MEA. Cannula tips were positioned in or near the MEA in the MEA AS-ODN groups and the MEA SAL groups.
DISCUSSION

Chronic bilateral infusion of an AS-ODN complementary to the translation start site of ERα mRNA into the MPO of gonadally intact male rats locally reduced the level of ERα in the MPO and suppressed mating behavior. Identical AS-ODN infusion into the MEA also locally reduced ERα levels but did not impair mating. Males infused with saline into either brain area mated robustly and had higher levels of ERα in comparison to those infused with AS-ODN. Specifically, in comparison to pretreatment levels of mating behavior and to the postoperative performance of control males, animals infused with AS-ODN in the MPO showed significant reductions in the number of mounts, intromissions, and ejaculations; whereas, males infused with AS-ODN in the MEA continued to mate normally.

This study tested the hypothesis that E2 acts through ERα in the MPO and the MEA to promote mating behavior. We predicted that chronic infusion of an AS-ODN complementary to ERα mRNA into either of these brain areas would knock down ERα expression in a site-specific manner in the MPO or the MEA and reduce mating. The hypothesis was only partially supported. Western blot analysis revealed a knock down of ERα proteins in both the MPO AS-ODN and MEA AS-ODN groups (as compared to the MPO SAL and MEA SAL groups) by approximately 4- and 6-fold, respectively. ERα ICC showed significantly reduced ERα-ir labeling in both the MPO and the MEA in males treated with AS-ODN as compared to those treated with saline in the same brain regions. The western blot analysis and ICC results are mutually reinforcing, suggesting that chronic infusion of the AS-ODN inhibited ERα expression in a site-specific manner in both the MPO and the MEA. Copulatory behavior was suppressed by MPO AS-ODN infusion, but not MEA AS-ODN infusion, where mating remained robust, indicating that the behavioral effects of the AS-ODN in the MPO must be attributed to the
specific effect of ERα reduction rather than to a non-specific, toxic effect of AS-ODN treatment. Additionally, because in this study we selectively disabled ERα production in the MPO or the MEA of gonadally intact male rats, it is likely that in the MPO and the MEA all remaining non-ERα steroid receptors were present and were exposed to naturally produced hormones in a physiologically relevant manner. Taken together, these results suggest that ERα mediates the responses of the MPO to E2 that are critical for mating behavior; whereas, ERα is not necessary in the MEA for sexual behavior to occur, and a different, non-ERα form of ER mediates the mating response to E2 in the MEA. Thus, E2 appears to exert its actions differently in the MPO and the MEA.

Previous research showed that E2 must act in the MPO [Clancy et al., 1995, 2000] and the MEA [Huddleston et al., 2003, 2006] for robust mating behavior to occur, and ERα has been reported to be the form of ER that is important for expression of mating [Rissman et al., 1997; Ogawa et al., 1998]. Physiological doses of either exogenous T or a combination of its androgenic and estrogenic metabolites, DHT and E2 respectively, maintain or restore sexual behavior in castrated male rats [Baum and Vreeburg, 1973; Larsson et al., 1973; Feder et al., 1974]. These studies are reinforced by additional findings with aromatase knockout (ArKO) mice in which ArKO males exhibited significantly reduced mating behavior [Matsumoto et al., 2003; Bakker et al., 2004] that was partially reversed by daily injections of either E2 or E2+DHT [Bakker et al., 2004]; however, these mouse studies do not address where specifically E2 acts in the brain. Blocking the conversion of T to E2 by site-specific infusion of the aromatase inhibitor Fadrozole in either the MPO [Clancy et al., 1995] or the MEA [Huddleston et al., 2006] decreases the sexual behavior of gonadally intact male rats; and, E2 implants to either the MPO
[Clancy et al., 2000] or the MEA [Huddleston et al., 2003] maintain mating behavior of intact male rats receiving systemic Fadrozole.

While it is clear that E₂ acts in the MPO and the MEA to promote mating behavior in male rats, the receptor and cellular mechanisms underlying the response to E₂ and how they contribute to male mating behavior are less well-understood. Presumably, most, if not all, of E₂’s actions are initiated through ER. However, the discovery of multiple ER subtypes [Kuiper et al., 1996; Thomas et al., 2005; Toran-Allerand, et al., 2002] raised questions as to which ER subtype(s) mediate mating and whether the numerous brain areas that contribute to sexual behavior use E₂ in an identical manner. Traditionally, it was thought that E₂ action was mediated by a single type of ER found within either the nuclear envelope [King and Greene, 1984] or the cytoplasm [Blaustein, 1992] of E₂-responsive cells. In 1996, a novel ER (now termed ERβ) was characterized that shared with the known ER (now termed ERα) both a high degree of sequence homology in the DNA (95%) and ligand (55%) binding domains (DBD and LBD, respectively) and similar affinities for most estrogenic and antiestrogenic substances [Kuiper et al., 1996, 1997]. Additionally, both ER subtypes exhibit similar transactivation properties, including a comparable ligand independent activation function 1 (AF-1) in the N-terminal region sensitive to the mitogen-activated protein kinase (MAPK) pathway [Kato et al., 1995; Tremblay et al., 1997] and a second ligand dependent activation function (AF-2) in the LBD that is enhanced by binding of coactivators, such as steroid receptor coactivator 1 (SRC-1) [White et al., 1997]. Interestingly, it has been reported that when gene transcription depends on both activation functions, activity of AF-1 in ERβ is negligible in the presence of ERα and that ERα activity dominates; however, if AF-1 is not required, the transcriptional activities supported by AF-2 of ERα and ERβ are similar [Cowley and Parker, 1999]; this may influence differential selective
transcription in brain areas expressing different levels of ER subtypes. Studies in female rats revealed differences in regional brain distribution of both ER subtypes and showed that brain regions implicated in the expression of mating behavior, including the MPO and the MEA, expressed both ER\(\alpha\) and ER\(\beta\) mRNA [Shughrue et al., 1997] and receptor proteins [Shughrue and Merchenthaler, 2001; Gréco et al., 2003]; moreover, levels of each receptor subtype also varied in subregional expression within individual brain areas further enhancing the regulatory potential of E\(_2\).

Following E\(_2\) binding, ERs form either homodimers [Kumar and Chambon, 1988] when a single ER subtype is present or both homo- and heterodimers [Cowley et al., 1997; Pettersson et al., 1997] in the presence of both ER\(\alpha\) and ER\(\beta\). These dimers may then act in either a classical manner, directly interacting with various estrogen response elements on the DNA of target genes to initiate transcription [Cowley et al., 1997; Pettersson et al., 1997; Tamrazi et al., 2002], or a nonclassical manner, involving protein-protein interactions with other transcription factor complexes bound to their response elements, such as activator protein 1 (AP-1), to indirectly initiate gene transcription [Jakacka et al., 2001]. Interestingly, nonclassical binding of ER in the AP-1 pathway resulted in transcription activation by ER\(\alpha\) and transcription inhibition by ER\(\beta\) [Paech et al., 1997; Jakacka et al., 2001], further suggesting that E\(_2\)’s actions may be modulated based on levels of ER subtypes present in target cells.

Rapid effects of E\(_2\) have been shown to be mediated via ER located on intracellular membranes [Revankar et al., 2005] or the plasma membrane [Razandi et al., 1999; Wade et al., 2001]. Although a membrane receptor has yet to be isolated and characterized, \textit{in vitro} studies indicate that membrane ER exist and are isoforms of distinct nuclear ER\(\alpha\) and ER\(\beta\) transcripts [Razandi et al., 1999, 2004]. In contrast, membrane actions of E\(_2\) have also been described that
are mediated by receptors unrelated to nuclear ER, mainly involving transduction via G-protein coupled mechanisms and activation of secondary signaling cascades which may or may not result in gene transcription [Nadal et al., 2000; Qui et al., 2003; Thomas et al., 2005]. Due to the complexities in defining the mechanisms of E₂ action, studies must be carefully structured to determine which ER form mediates any particular behavior, where the receptors are located on the target cells, and what the cellular consequences are when E₂ binds the receptor. The results from this study, together with recent reports, allow us to begin to answer these questions.

Both ERα-ir and ERβ-ir neurons occur in the rat MPO and MEA [Shughrue and Merchenthaler, 2001] and each region colocalizes mating induced Fos-ir [Gréco et al., 2003], suggesting that either ER subtype may contribute to mating behavior. Studies using estrogen receptor knockout (ERKO) mice suggest, however, that ERα is the form of ER that mediates copulatory behavior. Specifically, in gonadally intact mice lacking the ERα gene (αERKO), males show reduced intromission and no ejaculatory behaviors in comparison to wild type (WT) and heterozygous males [Ogawa et al., 1997]; whereas, other studies [Wersinger et al., 1997; Rissman et al., 1997, 1999] observed significant reductions in all components of copulatory behavior. In castrated αERKO male mice, androgen replacement did not restore the ejaculatory component of mating behavior, suggesting that ERα is important in the consummatory aspect of mating behavior [Ogawa et al., 1998]. In contrast to αERKO studies, observations in mice lacking the ERβ gene (βERKO) revealed few if any differences in the expression of sexual behaviors compared to WT mice [Ogawa et al., 1999], reinforcing the importance of ERα in the facilitation of male mating behavior. Nevertheless, male mice with a double knockout for both the ERα and ERβ genes (αβERKO) displayed no reproductive behaviors at all [Ogawa et al., 2000]. Together, these findings suggest a major role for ERα and a minor role for ERβ in the
regulation of male mating behavior. However, there are limitations to these reports. For example, studies showed increased levels of circulating sex hormones in ERKO mice [Eddy et al., 1996; Rissman et al., 1997], suggesting that knockout of a gene may result in second order effects. Findings by Apostolakis et al. [2002] suggest the possibility that activation of a secondary, compensatory system may mask the effects of total gene deletion. Questions also arise as to whether the observed effects are because the targeted gene product was unavailable during development or because it was unavailable during adulthood; and, most importantly, knockout studies do not tell us where in the brain gene inactivation is having its effect. Our use of antisense technology removes some of these difficulties because it allows for site-specific knockdown of a gene product during a restricted time periods rather than total ablation of the gene in the whole system.

We predicted that AS-ODN treatment to either the MPO or the MEA would attenuate sexual behavior, but copulation was significantly reduced by AS-ODN infusion of the MPO only; blockade of ERα expression in the MEA did not affect mating behavior. Moreover, reducing expression of ERα in the MPO significantly reduced all components of reproductive behavior, suggesting that ERα is necessary in the MPO for the expression of male rat sexual behavior. It is less clear which ER subtype is required in the MEA for mating, but these results suggest that ERα is not necessary in the MEA for male rat copulation to occur. These findings complement reports in female rats, in which mating-induced Fos-ir in the MPO was primarily coexpressed with cells that contained either ERα alone or both ERα and ERβ; whereas, in the MEA, mating induced Fos-ir was primarily coexpressed with cells that contained either ERβ alone or both receptor types [Gréco et al., 2003]. Together, these reports suggest that ERβ may mediate E2 responses required for mating in the MEA.
Although the present study does not address where ER are located on the target cells or what cellular mechanisms are employed for E₂ to exert its effects, studies using E₂ conjugated to bovine serum albumin (BSA-E₂), a large protein shown not to cross the plasma membrane thus restricting E₂ actions to the cell surface [Huddleston et al., 2007], are beginning to address these questions. Specifically, castrated DHT-maintained male rats receiving BSA-E₂ implants into the MPO mated normally [Huddleston et al., 2007]; whereas, males receiving BSA-E₂ implants into the MEA exhibited reduced sexual behavior [Huddleston et al., 2006]. These results suggest that a cell surface action of E₂ in the MPO is sufficient for the expression of mating behavior and, when combined with the present findings, suggest that this action occurs via an ERα protein associated with the plasma membrane, reinforcing that E₂ acts differently in the MPO than in the MEA.

The present findings help to delineate which ER subtypes are required in different areas of the brain where E₂ acts. Our findings suggest that, in the MPO, ERα is necessary for the expression of mating behavior; whereas, ERα is not necessary in the MEA for the expression of sexual behavior, suggesting that another ER subtype, perhaps ERβ, is important in the MEA. Thus, E₂ acts differently in different brain regions to mediate the expression of male rat copulatory behavior.
REFERENCES


