Steroid Sensitive Neurons and Male Rat Mating Behavior

Gloria Gradine Huddleston
MALE RAT MATING BEHAVIOR

by

GLORIA GRADINE HUDDLESTON

Under the Direction of Andrew N. Clancy

ABSTRACT

Male rat mating is a suite of individual behaviors mediated by the actions of two metabolites of testosterone (T), dihydrotestosterone (DHT) and estradiol (E2), on the brain. Individually, neither metabolite fully maintains or restores mating in castrated males, but both combined are as effective as T. Two hormone-responsive areas of the brain, the medial preoptic area (MPO) and the medial amygdala (MEA), are crucial for mating. These studies ask: by what mechanism(s) does E2 act in the MPO and MEA? We blocked the conversion of T to E2 in the MEA of intact male rats and sexual behavior was not maintained. We then infused antisense oligodeoxynucleotides (ODNs) to estrogen receptor-alpha (ER-α) mRNA bilaterally to the MPO or the MEA of intact male rats to block ER-α expression. ODN infusion of the MPO attenuated mating but infusion of the MEA had no effect. These results suggest that ER-α is the behaviorally relevant estrogen receptor (ER) in the MPO but not in the MEA. ER was originally described in the cytoplasm and nucleus of cells. Recently plasma membrane associated ERs (mER) have been reported. We conjugated E2 to Bovine Serum Albumin (BSA-E2), a large protein that will not penetrate the plasma membrane, thus restricting the action of E2 to mER, and chronically delivered it to the MPO and MEA. BSA-E2 maintained mating if
put in the MPO, but not in the MEA, suggesting a surface action of E$_2$ is sufficient in the MPO. The MPO and MEA are reciprocally connected and probably constitute elements of a larger, steroid-responsive neural network that mediates male mating behavior. To begin to describe this purported circuit, we injected Pseudorabies virus (PRV) into the prostate gland and dually labeled PRV-immunoreactive cells for ER or androgen receptors. We found dual labeling in a forebrain diencephalic circuit that includes the MPO, the medial preoptic nucleus, bed nucleus of stria terminalis, the zona incerta, the periaqueductal gray and other areas that presumably mediate both autonomic and motor aspects of male mating. Together, the results of these studies begin to elucidate locations and mechanisms of E$_2$ mediation of male sexual behavior.

INDEX WORDS: Androgen receptor, Aromatase, Bovine serum albumin, Dihydrotestosterone, Estradiol, Estrogen receptor, Mating behavior, Medial amygdala, Medial preoptic area, Oligodeoxynucleotide, Pseudorabies virus, Reproduction, Sexual behavior, Testosterone
STEROID SENSITIVE NEURONS AND MALE RAT MATING BEHAVIOR

by

GLORIA GRADINE HUDDLESTON

Major Professor: Andrew N. Clancy
Committee: H. Elliott Albers
Timothy J. Bartness
Sarah L. Pallas

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2006
ACKNOWLEDGEMENTS

Dr. Andrew N. Clancy
Dr. H. Elliott Albers
Dr. Timothy J. Bartness
Dr. Sarah L. Pallas
Rebecca A. Schorr
Jacquelyn Carrie Paisley
Marcelia Maddox
Fred Cawthon
Carol Callahan
Dr. Paul Franklin
Dr. Laine Giovanetto
Carolyn P. Huddleston
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 1: INHIBITION OF ESTROGEN RECEPTOR SYNTHESIS IN THE MEDIAL PREOPTIC AREA, BUT NOT THE MEDIAL AMYGDALA, REDUCES MALE RAT COPULATORY BEHAVIOR</td>
<td>8</td>
</tr>
<tr>
<td>Abstract</td>
<td>9</td>
</tr>
<tr>
<td>Introduction</td>
<td>10</td>
</tr>
<tr>
<td>Experimental Procedures</td>
<td>11</td>
</tr>
<tr>
<td>Results</td>
<td>16</td>
</tr>
<tr>
<td>Discussion</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 2: IMPLANTS OF ESTRADIOL CONJUGATED TO BOVINE SERUM ALBUMIN IN THE MALE RAT MEDIAL PREOPTIC AREA PROMOTE COPULATORY BEHAVIOR</td>
<td>35</td>
</tr>
<tr>
<td>Abstract</td>
<td>36</td>
</tr>
<tr>
<td>Introduction</td>
<td>37</td>
</tr>
<tr>
<td>Experimental Procedures</td>
<td>40</td>
</tr>
<tr>
<td>Results</td>
<td>44</td>
</tr>
<tr>
<td>Discussion</td>
<td>47</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

CHAPTER 3: EFFECTS OF ESTROGEN IN THE MALE RAT MEDIAL AMYGDALA: INFUSION OF AN AROMATASE INHIBITOR LOWERS MATING AND BOVINE SERUM ALBUMIN CONJUGATED-ESTRADIOL IMPLANTS DO NOT PROMOTE MATING................................................. 58
Abstract 59
Introduction 60
Materials and Methods 62
Results 67
Discussion 70

CHAPTER 4: COLOCALIZATION OF STEROID HORMONE RECEPTORS AND PSEUDORABIES VIRUS FOLLOWING INJECTION INTO THE PROSTATE GLAND OF MALE RATS...................................................... 86
Abstract 87
Introduction 88
Materials and Methods 90
Results 94
Discussion 97

CHAPTER 5: GENERAL DISCUSSION................................................. 122

LIST OF REFERENCES....................................................................... 128

APPENDIX: ESTRADIOL IN THE MALE RAT AMYGDALA FACILITATES MOUNTING BUT NOT EJACULATION...................................................... 161
Abstract 162
Introduction 163
Experimental Procedures 165
Results 169
Discussion 172
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
</tr>
<tr>
<td>4</td>
<td>121</td>
</tr>
<tr>
<td>5</td>
<td>183</td>
</tr>
</tbody>
</table>

1. Percent of Males That Copulated Within 1800 Seconds, by Behavior
2. Percent of Males That Copulated Within 1800 Seconds, by Behavior
3. PRV-ir and ER-ir
4. PRV-ir and AR-ir
5. Mean ± SEM Behavioral Indices and Numbers of Rats Exhibiting Specific Behaviors by Group
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mating behaviors (mean ± standard error of the mean) after AS-ODN or saline (vehicle) infusion of the MPO or MEA…</td>
</tr>
<tr>
<td>2</td>
<td>1D5 estrogen receptor immunocytochemistry of the medial preoptic area…</td>
</tr>
<tr>
<td>3</td>
<td>1D5 estrogen receptor immunocytochemistry of the medial amygdala…</td>
</tr>
<tr>
<td>4</td>
<td>Locations of cannula placements to the medial preoptic area…</td>
</tr>
<tr>
<td>5</td>
<td>Locations of cannula placements in the medial amygdala…</td>
</tr>
<tr>
<td>6</td>
<td>Mating behaviors (mean ± standard error of the mean) after BSA-E2, BSA + E2 (free E2) or BSA + cholesterol (vehicle) implants to the MPO…</td>
</tr>
<tr>
<td>7</td>
<td>Locations of cannula tips in animals implanted with BSA-E2 to the MPO, BSA + E2 (free E2) or BSA + cholesterol (vehicle)…</td>
</tr>
<tr>
<td>8</td>
<td>H222 ER-ir immunoreactivity in the MPO…</td>
</tr>
<tr>
<td>9</td>
<td>Mating behavior (mean ± standard error of the mean, SEM) after Fadrozole or saline infusion of the MEA…</td>
</tr>
<tr>
<td>10</td>
<td>Locations of cannula tips of animals infused with Fadrozole to the MEA and those infused with saline…</td>
</tr>
<tr>
<td>11</td>
<td>Mating behavior (mean ± SEM) after BSA-E2 or vehicle implants to the MEA…</td>
</tr>
<tr>
<td>12</td>
<td>Locations of implant tips of animals in the BSA-E2 conjugated group and the BSA + cholesterol group…</td>
</tr>
<tr>
<td>13</td>
<td>Method for preparation of maps describing the distribution of PRV-ir in the male rat brain…</td>
</tr>
<tr>
<td>14</td>
<td>Examples of neuronal labeling…</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Distribution of PRV-ir + ER-ir in a heavily-labeled case</td>
<td>106</td>
</tr>
<tr>
<td>16</td>
<td>Distribution of PRV-ir + ER-ir in a lightly-labeled case</td>
<td>110</td>
</tr>
<tr>
<td>17</td>
<td>Distribution of PRV-ir + AR-ir in a heavily-labeled case</td>
<td>114</td>
</tr>
<tr>
<td>18</td>
<td>Distribution of PRV-ir + AR-ir in a lightly-labeled case</td>
<td>117</td>
</tr>
<tr>
<td>19</td>
<td>Effects on the numbers (mean ± SEM) of mounts and intromissions and ejaculations of implanting E2 or vehicle bilaterally into the AMG of intact male rats receiving Fadrozole s.c</td>
<td>177</td>
</tr>
<tr>
<td>20</td>
<td>Location of implant tips in E2-implanted males and in vehicle-implanted</td>
<td>179</td>
</tr>
<tr>
<td>21</td>
<td>Photomicrographs of representative sections from the medial preoptic area and the medial amygdala from Fadrozole-treated male rats implanted bilaterally in the AMG with either E2 or vehicle</td>
<td>181</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHA</td>
<td>anterior hypothalamus</td>
</tr>
<tr>
<td>AMG</td>
<td>amygdala</td>
</tr>
<tr>
<td>ap</td>
<td>anterior posterior</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>antisense oligodeoxynucleotide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSA-E2</td>
<td>estrogen conjugated bovine serum albumin</td>
</tr>
<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CEA</td>
<td>central nucleus of the amygdala</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CTF</td>
<td>central tegmental field</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DM</td>
<td>dorsal medial hypothalamus</td>
</tr>
<tr>
<td>dv</td>
<td>dorsal ventral</td>
</tr>
<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>
</tbody>
</table>
### LIST OF ABBREVIATIONS (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ER-ir</td>
<td>estrogen receptor immunoreactive</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemical</td>
</tr>
<tr>
<td>id</td>
<td>inner diameter</td>
</tr>
<tr>
<td>IF</td>
<td>intromission frequency</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>-ir</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEA</td>
<td>medial amygdala</td>
</tr>
<tr>
<td>MEPO</td>
<td>median preoptic nucleus</td>
</tr>
<tr>
<td>mER</td>
<td>membrane associated estrogen receptor</td>
</tr>
<tr>
<td>MIS</td>
<td>missense oligodeoxynucleotide sequence</td>
</tr>
<tr>
<td>MF</td>
<td>mount frequency</td>
</tr>
<tr>
<td>ML</td>
<td>mount latency</td>
</tr>
<tr>
<td>ml</td>
<td>medial lateral</td>
</tr>
<tr>
<td>MNAR/PELP1</td>
<td>modulator of nongenomic actions of estrogen receptor / proline-, glutamic acid-, leucine-rich protein 1</td>
</tr>
<tr>
<td>MPN</td>
<td>medial preoptic nucleus</td>
</tr>
<tr>
<td>MPO</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>NDS</td>
<td>normal donkey serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>od</td>
<td>outer diameter</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PEI</td>
<td>post ejaculatory interval</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PH</td>
<td>posterior hypothalamus</td>
</tr>
<tr>
<td>PRV</td>
<td>pseudorabies virus</td>
</tr>
<tr>
<td>PVH</td>
<td>paraventricular nucleus hypothalamus</td>
</tr>
<tr>
<td>SAL</td>
<td>saline</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SCRAM1</td>
<td>scrambled oligodeoxynucleotide sequence 1</td>
</tr>
<tr>
<td>SCRAM2</td>
<td>scrambled oligodeoxynucleotide sequence 2</td>
</tr>
<tr>
<td>SNB</td>
<td>spinal nucleus of bulbocavernosus</td>
</tr>
<tr>
<td>SPFp</td>
<td>posterior subparafascicular nucleus of the thalamus</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor cofactor</td>
</tr>
<tr>
<td>st</td>
<td>stria terminalis</td>
</tr>
<tr>
<td>T</td>
<td>testosterone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TX</td>
<td>Triton-X</td>
</tr>
<tr>
<td>VMH</td>
<td>ventromedial hypothalamus</td>
</tr>
<tr>
<td>wt/wt</td>
<td>weight/weight</td>
</tr>
<tr>
<td>ZI</td>
<td>zona incerta</td>
</tr>
</tbody>
</table>
INTRODUCTION

In vertebrates, the expression of male copulatory behavior depends on testicular testosterone (T) and its metabolites. Gonadal hormones not only maintain peripheral structures, but also act on the central nervous system, orchestrating a suite of individual behaviors that range from premating rituals to successful insemination. For thousand of years, farmers and sheiks have utilized the physiological changes induced by castration. In our modern world we have developed the ability to manipulate hormones to give desired effects (birth control, fertility, libido enhancement and inhibition), yet the exact action of a single hormone or the specific mechanism of any hormone’s effect on the brain and sexual behavior is not understood. Sexual behavior is a complex mixture of motivation, learned behavior, natural instinct, and rapidly changing physiological states. Presumably, the “conductor” of this “orchestra” is the central nervous system. Hormones cause cellular changes in neurons of the brain and spinal cord coordinating both the physiological and behavioral aspects of mating, but whether these changes are activational, permissive, or inhibitory is unknown. The studies presented here do not fully answer these questions and in fact, raise more questions for future research. Nevertheless, these findings will hopefully add pieces to the picture that will one day explain the activation affects of hormones on the brain and their transduction into male sexual behavior.

Studies of the central regulation of any behavior, by necessity, require identification of the anatomical locations that govern the behavior as well as the neuropharmacological functioning of these sites. These studies are concerned with the
actions of gonadal steroids on the MPO and MEA, two sites that are important for male sexual behavior. T is enzymatically metabolized to dihydrotestosterone (DHT) by 5-α-reductase [90] and to estradiol (E2) by aromatase [108]. Numerous hormone manipulation studies have suggested that individually, neither DHT nor E2 is as effective in maintaining or restoring male copulatory behavior as is a combination of the two [158] or as is T. Today, there is a consensus that hormonal control of male mating behavior depends on both metabolites of T and probably T itself.

Lesion studies have suggested that several brain areas such as the medial preoptic area (MPO), the bed nucleus of the stria terminalis (BST) the central tegmental field (CTF) and the amygdala, among others contribute to the expression of copulatory behavior [63, 97]. The MPO, believed to be center for integration, is crucial for male mating behavior. Lesions, either electrical [28, 78, 80] or chemical [56, 80], of the MPO attenuate mating, whereas stimulation of the MPO, either electrical [87, 153] or chemical [43, 44], promotes it. Knife cut experiments severing various connections afferent and efferent to the MPO have suggested that the efferent connections from the MPO to the median forebrain bundle are crucial to the display of mating behavior [14]. In every vertebrate studied to date, the absence of a functional MPO results in an inability to mate. Lesions of the BST and/or the stria terminalis (st; a fiber tract that connects the amygdala with the MPO) interrupt male mating but to a lesser extent than do lesions of the MPO. Specifically, BST and st lesions generally affect the cadence of mating, resulting in increased frequencies of behaviors preceding ejaculation and longer latencies until ejaculation occurs [45, 48, 150, 177]. Electrical lesions of subnuclei of the amygdala,
namely the medial amygdala (MEA) cause severe impairment of mating behavior [71] while other reports suggest a temporal or timing disruption similar to lesions of the BST [80]. Nevertheless, the importance of the MEA in male mating behavior cannot be discounted. In all probability the MPO and the MEA function as a unit by controlling and integrating different aspects of the behavior [40, 74, 171].

Fos, the protein product of the early immediate gene *c-fos*, is accepted as a correlational marker for neuronal metabolic activity [131]. Supporting previous studies, mating-induced Fos-immunoreactivity (-ir) is increased in brain areas known to mediate mating, namely the MPO, BST, MEA and CTF [8, 36, 70]. Moreover, Fos-ir associated with mating is colocalized with either AR-ir or ER-ir alone, or both AR-ir and ER-ir in neurons of these same brain areas [51]. Anatomical tract tracing studies show reciprocal connections between these areas [18, 38, 39, 52, 136], raising the possibility that, in males, there is an interconnected, steroid-responsive neural network that mediates male mating behavior, similar to the network that governs the lordotic reflex in female rats [41]. In all probability, such a network does exist, but little is known about the specifics of the steroid-responsiveness. Systemic T given to castrated male rats is capable of maintaining [163] or reinstating [43] male sexual behavior, however, some studies suggest that it is the conversion of T to E$_2$ that stimulates mating rather than T alone [83, 84, 141]. Furthermore, sexual behavior is reinstated in castrated males that receive T or E$_2$ implants to the MPO [43, 44], and exogenous T plus anti-estrogens are insufficient to reinstate behavior in castrated males [83], suggesting that estrogen might play a larger role in the expression of male sexual behavior than previously imagined.
Previous studies from our laboratory using subcutaneous (s.c.) administration of Fadrozole (CGS-16949A, CIBA-Geigy Corporation, Summit, NJ), an aromatase enzyme inhibitor, significantly decreases the conversion of $[^3H]T$ to $[^3H]E_2$ in male rat hypothalamic and amygdaloid nuclear fractions [12]. Moreover, administering Fadrozole s.c. (but not vehicle) to castrated, T-maintained male rats significantly decreases mating behavior, and subsequent administration of E$_2$ partially restores some copulatory behaviors [12]. Chronic intracranial infusion of Fadrozole (but not saline) bilaterally to the MPO of intact male rats also significantly decreases mating [32]. Conversely, mating behavior is maintained in intact males given chronic Fadrozole s.c. (to block T aromatization throughout the brain) together with bilateral MPO implants of E$_2$ (but not vehicle) [33]. Clearly, the action of E$_2$ in the MPO is crucial for the display of male copulatory behavior, but what about the MEA? To examine the role of E$_2$ in the MEA, we used the same experimental approach as described above. In Chapter 2, Fadrozole was chronically and bilaterally infused to the MEA of intact male rats, which were subsequently tested with receptive females and scored for the expression of sexual behavior. The results of the converse experiment are reported in the Appendix: chronic Fadrozole s.c. together with bilateral E$_2$ implants (but not vehicle implants) aimed at the MEA maintain mounting and intromissive behaviors in intact males. Ejaculations however, are not maintained.

Obviously, E$_2$ also acts in the MEA to mediate male sexual behavior, but the exact mechanism(s) of E$_2$ action is misunderstood. Historically, ER (found in both the MPO and the MEA) was known to act as a transcription factor, regulating gene
expression [64, 68, 93, 164]. Recently, there have been reports of ER associated with the plasma membrane (mER) [88, 101, 148] or intracellular membranes [128, 176] that act rapidly initiating a cascade of cell-signaling effects that might or might not culminate in gene transcription [160]. In 1996, a novel ER was described [76] as ER-β, causing the previously discovered ER to be called ER-α. Behavioral studies suggest that mice genetically altered to be deficient in the expression of ER-α do not mate [112, 129] whereas mice with ER-β deficiencies mate at levels that are not significantly different from wild type [111]. We have previously shown that E2 functions in the MPO and MEA to promote mating behavior. We hypothesized that if, as suggested, ER-α is behaviorally relevant for mating, then selective removal of ER-α from the MPO and MEA of male rats should reduce mating. To test this hypothesis we used bilateral chronic infusion of antisense oligodeoxynucleotides complementary to the start translation site of ER-α mRNA to knockdown the expression of ER-α in the MPO and MEA of intact male rats and tested them with sexually receptive females.

Because of the numerous recent reports of mER cell-signaling we wondered if these signaling cascades were important in the expression of specific behaviors. Initially, such rapid signals were considered to be “non-genomic” in nature; however, research is beginning to suggest that these rapid signals and genomic effects are not mutually exclusive [160]. Hormones can be conjugated to Bovine Serum Albumin (BSA - a large protein that does not cross the cellular plasma membrane) and used to stimulate only those receptors associated with the membrane. Thus, conjugated steroids are an excellent tool for studying the effects of membrane-associated receptors. Surprisingly, most of the
studies using conjugated steroids have been conducted in vitro. Reports of in vivo studies of the behavioral effects of membrane-associated receptors are rare, however it has been shown that progesterone conjugated to BSA, when administered to the ventral tegmental area, elicits lordosis in estrogen-primed female rats [47]. Therefore, we used E₂ conjugated to BSA (BSA-E₂) to explore the behavioral effects of mER via chronic bilateral delivery to either the MPO or MEA of castrated, DHT-maintained male rats.

Finally, if a steroid-responsive neural network for male sexual behavior exists, then, based on lesion, autoradiography, Fos-ir and hormone manipulation studies, brain areas previously described, such as the MPO, BST, MEA and CTF, should be components of the circuit. This circuit should show steroid hormone sensitivity and connectivity to peripheral structures involved in mating and insemination. Recently a series of studies have described a spinal ejaculation generating center in the lumbar spinal cord [35, 149]. Ejaculation, however is a reflex (as is lordosis), and its expression presumably can be influenced by supraspinal elements in the brain. Moreover, because not all sexual behavior culminates in ejaculation, we hypothesized that there must be a supraspinal circuit for mating behavior that integrates exogenous sensory cues with the motor output needed to initiate mating behavior, thus synchronizing and coordinating sexual response.

To test the hypothesis we injected an attenuated form of the pseudorabies virus (PRV), the Bartha’s K strain, a transneuronal, retrograde tract tracer, into a peripheral structure that is activated during mating, the prostate gland. When this viral strain is introduced peripherally, it is taken up by autonomic motor neuron terminals
Inside the infected neuron, the virus self-replicates and is transferred across synapses in a retrograde fashion to presynaptic axon terminals. Provided there is no cell lysis, the process is repeated and viral infection spreads from neuron-to-neuron in a chain-like fashion [19, 20] such that, with anti-PRV ICC, an entire multisynaptic neural network that presumably controls the functions of the peripheral structure can be visualized. We elected to infect the prostate gland with PRV because the expulsion of prostatic fluid is temporally synchronized with the ejaculatory behavioral pattern, which suggests that activity in a common, supraspinal circuit may trigger both the expulsion of prostatic fluid and the behavioral patterns associated with mating behavior. We reasoned that PRV would infect the central neural network underlying mating behavior and we predicted that this network would be composed of steroid-sensitive neurons. Brains were dually processed for the expression of PRV-ir and either ER-ir or AR-ir and we anticipated finding a high proportion of dual-labeled neurons in brain areas known to mediate male rat sexual behavior.
CHAPTER 1

INHIBITION OF ESTROGEN RECEPTOR SYNTHESIS IN THE MEDIAL PREOPTIC AREA, BUT NOT THE MEDIAL AMYGDALA, REDUCES MALE RAT COPULATORY BEHAVIOR


Portions of this chapter are based on the Masters Thesis of J. C. Paisley.
ABSTRACT

Male rat sexual behavior depends on the actions of gonadal hormones on steroid-sensitive neurons in the brain. Mating-induced Fos-immunoreactivity (ir) is colocalized with estrogen receptors (ER) in neurons of the medial preoptic area (MPO) and the medial amygdala (MEA), and inhibiting the aromatization of testosterone to estradiol (E$_2$) in either of these areas impairs mating, whereas E$_2$ implants to either area facilitate it. Because several studies suggest that ER$_{\alpha}$ is the behaviorally relevant form of ER, we hypothesized that E$_2$ acts via ER$_{\alpha}$ in the MPO and MEA to mediate mating. To test the hypothesis, we used bilateral infusions of an antisense oligodeoxynucleotide (AS-ODN) complementary to ER$_{\alpha}$ mRNA, to either the MPO or the MEA of intact male rats, thus blocking the synthesis and expression of ER$_{\alpha}$ in those brain areas. Infusion of AS-ODN significantly reduced expression of ER$_{\alpha}$-ir in both the MPO and MEA relative to control infusions. Furthermore, males receiving AS-ODN to the MPO exhibited significant deficits in the numbers of mounts, intromissions and ejaculations compared to controls. However, males receiving AS-ODN to the MEA continued to mate normally. These results suggest: (i) that ER$_{\alpha}$ is the behaviorally relevant form of ER in the MPO but not the MEA and (ii) that E$_2$ has differential actions in these two brain areas.
INTRODUCTION

Lesion [28, 56, 78], stimulation [87, 153] and hormone manipulation studies [43, 44] have all shown that a functional medial preoptic area (MPO) is crucial for male rat mating behavior, moreover, mating-induced Fos immunoreactivity (-ir) is colocalized in neurons of the MPO that contain androgen receptors (AR), estrogen receptors (ER) or both AR and ER [51]. In the brain, testicular testosterone (T) is enzymatically converted to dihydrotestosterone (DHT) via 5-alpha reductase [90] and to estradiol (E2) via aromatase [108]. Blockade of the conversion of T to E2 via Fadrozole, (a non-steroidal aromatase enzyme inhibitor, CGS-16949A, CIBA-Geigy Corporation, Summit, NJ) administered either systemically [12] or locally into the MPO [32] attenuates mating behavior, an effect that is reversed by systemic E2 [12]. Furthermore, implants of E2 confined to the MPO maintain mating behavior in male rats given systemic Fadrozole [33]. Thus, estrogenic metabolites of T, acting in the MPO, are crucial for the expression of male mating.

The medial amygdala (MEA) also colocalizes ER and mating-induced Fos-ir [51]. Reproductively significant olfactory [110], chemosensory [37, 110] and somatosensory [8, 52] stimuli are received and processed in the MEA, which is heavily reciprocally interconnected with the MPO [18, 136]. Electrolytic lesions of the MEA [57] or knife cuts [73, 150] of pathways connecting the MEA and MPO produce severe deficits in mating behavior. Moreover, blocking T aromatization in the MEA reduces sexual behavior [60], and E2 implants confined to the MEA maintain mounts and intromissions, but not ejaculations [59] in male rats administered Fadrozole systemically. Although a
functional MPO appears to be sufficient for the expression of copulation, it is likely that the MEA and MPO operate together (perhaps as a unit) to mediate copulatory behavior [72] and that the MEA facilitates mating.

It is evident from these studies that responses to E\textsubscript{2} occur in the MPO and MEA leading to normal male mating behavior. The mechanism(s) of estrogen action, however, have yet to be clarified, although recent research suggests that hormone-bound neuronal steroid receptors exert their effects by acting in conjunction with one or more steroid receptor cofactor (SRC) proteins. Inactivation of these SRCs [27, 103] or ER itself [91] via intracranial delivery of antisense oligodeoxynucleotides (ODNs) complementary to their respective mRNAs interferes with organizational and activational effects on mating.

Studies using genetically altered mice in which ER\textsubscript{α} was knocked out suggest that the behaviorally relevant ER is ER-\textalpha [112, 129]. We hypothesized that, in the male rat MPO and MEA, ER-\textalpha is the behaviorally significant form of ER and that selective removal of ER-\textalpha individually from these brain areas would reduce mating behavior. We tested this hypothesis by bilateral chronic infusion an antisense ODN complementary to the start translation site of ER-\textalpha mRNA locally into the MPO or the MEA, to knockdown the expression of ER-\textalpha and monitored changes in copulatory behavior.

**EXPERIMENTAL PROCEDURES**

*Animals.*

Male and female Sprague-Dawley rats, at least 90 days of age, were obtained from Charles River Laboratories. Animals were housed in polycarbonate cages, 22 x 44
12 cm, with free access to food and water in the university vivarium on a 14:10 hour reverse light:dark cycle (lights off at 0930 hours). Males were housed two per cage until the day of surgery and thereafter, they were single housed. 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).

Female rats were anesthetized with isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an oxygen exchange rate of 500 cc per minute and transferring them to a nosecone dispensing 2-3% gas at 500 cc per minute oxygen exchange rate, ovariectomized and implanted s.c. with a 6 mm Silastic capsule filled with crystalline E2. Thereafter, all females were allowed at least seven days recovery time before the start of behavioral testing. On testing days, females received 1.0 mg progesterone s.c. (1mg progesterone/0.2 ml sesame oil) 4-6 hours before being paired with males.

Male rats were screened for sexual behavior in four weekly 30 minute 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).

Each male was anesthetized with isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an oxygen exchange rate of 500 cc per minute and transferring them to a nosecone dispensing 2-3% gas at 500 cc per minute oxygen.
exchange rate and stereotaxically implanted bilaterally with 28 gauge stainless steel, ethylene oxide sterilized, cannulae aimed at either the MPO (level skull coordinates: ap = -0.5 mm, ml = ± 0.75 mm, dv = -9.0 mm) or the MEA (level skull coordinates: ap = -3.2 mm, ml = ± 3.5 mm, dv = -9.2 mm) [146]. Each cannula was connected via Tygon microbore tubing to an osmotic minipump (Alzet, model 2004) implanted subcutaneously 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 5-7 days surgical recovery, weekly 30 minute sexual behavior testing 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 evaluated to assure there were no pump failures; none occurred.

**Antisense Oligodeoxynucleotide Preparation.**

Experimental animals were infused via minipumps with 25-mer ODN sequence (5’-GTGAAGGGTCATGGTCATGGTGAGT-3’) antisense to the translation start codon for rat ER-α [143]. Lyophilized ODN (6290 μg, Oligos Etc. Inc., Wilsonville, OR) was resuspended 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/12 μl, 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 hours 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 a scrambled sequence (SCRAM1: 5’ – CTCTACACATCGTGGATCGTGACTG – 3’, MPO N=5, MEA N=5; SCRAM2: 5’ – CTAGGTCTAGCTGTCCACACGTGAG – 3’, MPO N=7) or a missense sequence (MIS: 5’ – GTGTAGGGTTATGGTCATTGTAAGT – 3’, MPO N=9). These altered sequences were not complementary to ER mRNA or any other known rat 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 physiological or behavioral abnormalities which resulted in death or required humane euthanasia. Therefore, further attempts to develop this type of control were abandoned for ethical considerations.

Sexual behavior tests.

During preoperative sexual screening and postoperative mating tests, each male was paired with a sexually receptive female for 30 minutes in a 22 x 44 x 50 cm testing arena under dim red light. Tests began about 3 hours 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 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 additional mounting or intromission occurred.

**Histological verification.**

When the testing period was concluded, males were euthanized with an overdose of sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially for 5 minutes 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 0.1M PB for approximately 48 hours. Coronal frozen brain sections (40 μm) through the diencephalon were collected into 0.1 M PB, mounted on clean gel-albumin coated slides, and stained with Toluine blue to verify cannula placement.

**Estrogen Receptor Immunocytochemistry.**

Coronal sections from animals in the four groups were processed concurrently with the same aliquots of antibodies and immunoreagents and were incubated first in a blocking solution containing 5% normal donkey serum (NDS) in 0.1 M phosphate buffer (PB), pH = 7.4 for 1 h at room temperature and second with the monoclonal 1D5 anti-ER antibody for 48 h at room temperature. The 1D5 anti-ER antibody (Zymed Laboratories, catalogue # 18-7149, lot # 40380301) 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 at room temperature with a donkey antimouse 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 at room temperature in an avidin-biotinylated peroxidase complex (Vector Elite Standard ABC kit, catalogue # 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 at room temperature in a solution containing 0.2 mg/ml diaminobenzidine (Sigma) plus 0.1 μl/ml 30% H₂O₂ (Sigma) dissolved in 0.1 M PB.

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 [69]. The Mann-Whitney U test [133] was used to analyze immuno-cytochemical data. Two-tailed probabilities are reported unless otherwise stated.

RESULTS

Male Sexual Behavior.

We tested the hypothesis that chronic AS-ODN infusion would reduce mating by observing copulatory behavior. Highly significant group differences emerged during the postoperative period. 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 on all trials (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 mainly during the postoperative trials.

In a follow-up analysis, groups MPO SAL, MEA AS-ODN and MEA SAL were pooled because post hoc comparisons from the analysis above indicated that these 3 groups were statistically indistinguishable on every behavioral measure on all postoperative trials; 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, whereas robust mating behavior was expressed on all postoperative trials by animals in the combined control
group, as reflected in all behavioral measures. Specifically, during postoperative trials, the resulting 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 comparisons between the 2 groups 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 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, between the preoperative and postoperative periods, considering the same sets of trials, 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. Moreover, considering 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.**

We tested the hypothesis that chronic AS-ODN infusion would knockdown ER-α expression by performing ER-α ICC. Infusion of the MPO (FIGURE 2) or MEA (FIGURE 3) with AS-ODN resulted in suppression of ER-ir neuronal labeling in the MPO or MEA, respectively, whereas saline infusion of either brain area was accompanied by appreciable ER-ir labeling. Specifically, significantly fewer ER-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 ER-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 ER-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) or the MEA AS-ODN and MEA SAL groups (30.54 ± 9.32 and 58.33 ± 11.14, respectively).

**Cannula Placements.**

Locations of cannula placements to the MPO are indicated in FIGURE 4 and those to the MEA in FIGURE 5. Cannula tips were positioned in or near the medial preoptic area or medial amygdala, respectively, in almost all cases in all groups of animals.

**DISCUSSION**

Chronic bilateral infusions of the MPO with an ODN complementary to the start translation site of ER-α mRNA knocked down expression of ER-α and impaired mating in gonadally intact male rats, whereas infusions of the MEA also knocked down expression of ER-α, but did not impair mating. Specifically, males in the ODN-MPO group exhibited severe deficits in mating compared to vehicle controls or to their pretreatment levels, but rats in the ODN-MEA group continued to mate at levels that were not significantly different from vehicle controls or their pretreatment levels. Because E2 action in these brain areas is crucial for mating [12, 32, 33, 59, 60] and because ER-α is reported to be the behaviorally relevant form of ER [112, 129], we
hypothesized that the knockdown of ER-α in either brain area would suppress mating. This hypothesis was partially supported: ER-α mediates the responses to E₂ in the MPO that promote mating behavior, but ER-α is not necessary in the MEA for sexual behavior to occur. These results suggest that the mechanisms by which E₂ exerts its effects differ in the MPO and MEA, thus suggesting differential actions of E₂ in individual brain areas.

Traditionally, ER has been described as a nuclear [68, 76] and/or cytoplasmic protein [10] that upon binding E₂ acts as a transcription factor for protein synthesis. At present, there are two known forms of ER, ER-α and ER-β and both are believed to act as transcription factors, although some reports differ [25]. The receptors are similar in the ligand binding domains [165] but dissimilar enough in homology, binding kinetics and transactivation properties that some have suggested that each functions in a unique manner [26, 115]. Razandi et al. reported that both receptors arise from a single transcript [127] and might be a product of variant splicing. The receptors dimerize upon binding E₂ [147], creating homodimers if a single receptor type is present in the target cell or homo- and heterodimers if both ER-α and ER-β are present; this may influence differential selective transcription. Recently, a novel form of ER has been described that functions to integrate signal transduction pathways [128, 148] and we now know that this novel receptor, as well as the classical α and β forms, might be associated with the cell surface, causing signaling cascades that might or might not culminate in gene transcription. Because the actions of E₂ are complex, studies must be carefully structured to define the behaviorally relevant actions of E₂; they must address questions such as which receptors are behaviorally relevant, how ERs cause cellular responses and where
the receptors are found in the target cells. The results from the present study, together with recent reports, allow us to begin to answer these questions: we conclude that the behaviorally relevant ER in the MPO is ER-\(\alpha\).

Steroids act in conjunction with SRC proteins that enable ligand-bound receptors to alter transcriptional activity [5]. Such genomic actions seem to be necessary for mating behavior to occur, because infusion of transcription inhibitors attenuates mating [93]. It has been suggested that E\(_2\) acts on the genome as a “chemical switch” [137] to promote the synthesis of enzymes for the production of neurotransmitters or other neurochemicals that mediate mating. Novel approaches are being used to discover the mechanism(s) of steroidal action on neurons \(\text{in vivo}\). Infusion of antisense ODNs that inhibit transcription by blocking SRCs interfere with brain sexual differentiation [91, 104], and the activation of sexual behaviors [27, 103].

Studies with E\(_2\) conjugated to Bovine Serum Albumin (BSA-E\(_2\)) are beginning to address the question of where E\(_2\) acts at the level of the target cell. BSA-E\(_2\) does not enter into the interior of the cell but has been shown to act solely at the cell surface [161], thereby stimulating plasma membrane-associated receptors. When implanted into the MPO of castrated, DHT-treated male rats, BSA-E\(_2\) maintained mating behavior; conversely, mating was attenuated in an identical group that received BSA-E\(_2\) implants to the MEA, which suggests that (i) E\(_2\), acting at the cell surface of MPO neurons, is sufficient for the expression of mating behavior and (ii) that E\(_2\) acts differently in the MPO and MEA. The results of the present study, together with the BSA-E\(_2\) findings, suggest that the behaviorally relevant ERs are associated with the cell surface in the MPO.
and that these ER are ER-α, consistent with reports that the membrane-associated ER is some form of ER-α [88, 101, 159]. In view of the likelihood that genomic activation is necessary for the behavioral response to E2, there might be at least two ways that E2 can activate the genome: through a classical pathway that involves intracellular ER or through a transduction mechanism that is coupled to a plasma membrane-associated ER. Future studies need to determine if there is only one or more than one behaviorally relevant signaling pathway. If multiple or parallel pathways exist, do they eventually culminate in the same result and can one pathway be substituted for another?

In conclusion, the major findings from this study are that ER-α mediates the sexual response to E2 in the MPO and that E2 acts differently in the MPO and MEA. Because we employed bilateral, site specific soft-tissue infusion of ODN (instead of intra-ventricular infusion) we can begin to develop a specific model of E2 action. The model is based on three premises taken from the present and other findings: (i) the behavioral response to E2 is mediated in the MPO by ER-α, (ii) ER-α can associate with the plasma membrane and (iii) a cell surface action of E2 in the MPO is sufficient for mating, therefore, the behaviorally relevant ER in the MPO is a plasma membrane-associated ER-α. Although E2 is important in the MEA for male mating to occur, ER-α does not mediate this response; instead, an intracellular, non-α ER appears to govern the response to E2 in the MEA.
ACKNOWLEDGEMENTS

This work partially fulfills the Department of Biology, Georgia State University Doctor of Philosophy degree requirements for G. G. Huddleston and the Masters of Science degree requirements for J. C. Paisley and was supported by National Science Foundation grant IBN 9876754 to the Center for Behavioral Neuroscience.
FIGURE 1
Mating behaviors (mean ± standard error of the mean) after AS-ODN or saline (vehicle) infusion of the MPO or MEA. Males infused with vehicle to the MPO or MEA mated normally as did rats infused with AS-ODN to the MEA, whereas those receiving AS-ODN to the MPO virtually ceased mating. Blackened symbols indicate the trials where *post hoc* statistical comparisons revealed that the MPO AS-ODN group differed significantly from the other groups (p < 0.05, two-tailed).
FIGURE 1

MPO AS-ODN (N = 6): p = n.s. & p < 0.05
MPO SAL (N = 8):
MEA AS-ODN (N = 6):
MEA SAL (N = 7):
FIGURE 2
1D5 estrogen receptor immunocytochemistry of the medial preoptic area. Sections through the MPO and arcuate nucleus from males in the MPO AS-ODN and MPO Saline groups were processed concurrently with the 1D5 anti ER-α antibody. ER-ir labeling did not occur in the MPO of males in the MPO AS-ODN group (top left) but was expressed normally in the MPO of males in the MPO Saline group (top right) and in the arcuate nucleus in all cases (bottom row) suggesting that infusion of AS-ODN blocked expression of ER-α in the MPO.
FIGURE 2
FIGURE 3

1D5 estrogen receptor immunocytochemistry of the medial amygdala. Sections through the MEA and arcuate nucleus from males in the MEA AS-ODN and MEA Saline groups were processed concurrently with the 1D5 anti ER-α antibody. ER-ir labeling did not occur in the MEA of males in the MEA AS-ODN group (top left) but was expressed normally in the MEA of males in the MEA Saline group (top right) and in the arcuate nucleus in all cases (bottom row) suggesting that infusion of AS-ODN blocked expression of ER-α in the MEA.
FIGURE 3
FIGURE 4

Locations of cannula placements to the medial preoptic area. Cannula tips were positioned in or near the MPO in almost all cases in the MPO AS-ODN and MPO Saline groups.
FIGURE 5

Locations of cannula placements in the medial amygdala. Cannula tips were positioned in or near the MEA in almost all cases in the MEA AS-ODN and MEA Saline groups.
FIGURE 5

MEA AS-ODN

MEA SALINE
CHAPTER 2

IMPLANTS OF ESTRADIOL CONJUGATED TO BOVINE SERUM ALBUMIN IN THE MALE RAT MEDIAL PREOPTIC AREA PROMOTE COPULATORY BEHAVIOR

Gloria G. Huddleston, Jacquelyn C. Paisley and Andrew N. Clancy, Neuroendocrinology

ABSTRACT

The expression of mating behavior in male rats is dependent on estrogen-responsive neurons in the medial preoptic area (MPO). Previous reports showed that mating is attenuated if the aromatization of testosterone to estradiol (E2) is blocked in the MPO [32] and that mating is maintained by MPO E2 implants [33]. However, the mechanisms by which E2 exerts its action are not fully understood. It had been thought that E2 acted exclusively by binding to nuclear estrogen receptors to exert its effects; however, recent reports suggest that E2 also binds to membrane-associated receptors activating downstream intracellular cascade responses. In this study, we asked if an action of E2 at the cell surface is sufficient to support mating behavior. Therefore, either vehicle, E2, or E2 conjugated to Bovine Serum Albumin (BSA-E2: a complex of E2 and a large protein that will not cross the plasma membrane, thereby restricting the action of E2 to cell-surface signaling) was chronically administered bilaterally to the MPO of castrated, dihydrotestosterone-treated male rats. Mating behavior was supported by MPO BSA-E2 implants, suggesting that E2 operates in the MPO via a cell-surface mechanism to facilitate male rat mating behavior.
INTRODUCTION

In mammals, gonadal hormones act on both central and peripheral structures to promote mating. Testicular testosterone (T) may be converted enzymatically in target tissues into at least two metabolites: dihydrotestosterone (DHT) via 5-alpha reductase [90] and estradiol (E2) via aromatase [108]. These metabolites, and probably T itself, maintain peripheral sexual organs; moreover, their actions on steroid sensitive central neurons are necessary for male sexual behavior to occur [151]. It is likely that these central neurons form a pathway or network in the male rat brain [43] analogous to the pathway governing the lordotic reflex in female rats [41, 119]. Under the influence of steroids, this pathway transduces or enables the integration of motivational stimuli and the execution of mating.

In every vertebrate species studied to date, a functional response to gonadal steroids takes place in the medial preoptic area (MPO) that is essential for male reproductive behavior to occur. The MPO receives processes and integrates sexually relevant inputs [134] and transmits information to brain areas that regulate autonomic, somatomotor and motivational states necessary for mating [136]. Both electrolytic [28, 78, 80] and chemical [56, 80] lesions of the MPO result in severe deficits in male mating, as do strategically placed knife cuts severing efferent pathways of the MPO [14, 86]. Electrophysiological studies of MPO neurons have shown increased activity in response to T [120, 175] and E2 [17, 65], and it has been shown that both androgens [96], and E2 [44] are required for robust mating behavior. Supporting these ideas, Greco et al., showed that mating-induced Fos-ir (the protein product of the c-fos gene, an accepted
marker of metabolic activity in neurons [131]) is colocalized in neurons with androgen receptors (AR), estrogen receptors (ER) or both receptor types in brain areas known to contribute to the expression of male mating behavior, including the MPO, medial amygdala (MEA), bed nucleus of stria terminalis, and central tegmental field [51]. Hormone manipulations within these areas can modify sexual responses and compromise the functioning of the purported neural network that governs male mating behavior.

In studying the effect(s) of E2 on neurons in this network, we showed that systemic administration of Fadrozole (CGS-16949A CIBA-Geigy Corporation, Summit, NJ), a non-steroidal aromatase enzyme inhibitor that blocks the conversion of T to E2, results in severe copulatory deficits in castrated, T-maintained male rats, which exogenous E2 partially reverses [12]. Local administration of Fadrozole to the MPO of intact males significantly attenuates mating but vehicle MPO implants do not [32]. Conversely, intact males given systemic Fadrozole and MPO vehicle implants suffer severe deficits of mating, whereas those receiving MPO E2 implants continue to mate [33].

Clearly, the presence of E2 in the MPO of male rats is crucial for the display of mating behavior; the mechanism of E2 action(s), however, is less well understood. Traditionally, it had been believed that E2 either binds to a cytoplasmic receptor that translocates to the nucleus or to an intranuclear receptor, ultimately interacting with DNA and serving as a transcription factor for the production of protein products via mRNA synthesis [64, 68, 164]. Infusion of transcription inhibitors attenuates mating [93], as does infusion of antisense oligonucleotides targeting steroid receptor co-activators [27],
suggesting the necessity of a genomic response to E2. Although reports of rapid effects of E2 on neurons and cells of other tissue have been long been reported [102, 121], a plethora of recent reports, as well as reports of a novel G-protein membrane-associated ER [128, 148], imply that rapid cellular responses may result from E2 binding to membrane-associated receptors (mER). Though some differences between reports remain, it is generally accepted that rapid effects of E2 have been shown, as demonstrated by intracellular increases of phosphorylated CREB [180], phosphorylated MAPK [161], cAMP [102], calcium [21, 24], or other pathways, as well as changes in membrane permeability [77, 121], and it is believed that these rapid effects might [180] or might not [77] involve genomic action. There are differing theories as to the nature of these mERs: (i) they might or might not be inserted into, or tethered to, the plasma membrane, (ii) they might aggregate at its inner surface or (iii) they might shuttle from the cytoplasm to the plasma membrane in the presence of E2. These ideas raise questions about how E2 acts. Is a surface action of E2 sufficient to cause physiological responses that affect behavior, or must E2 act intracellularly to have an effect on behavior, or both? We hypothesized that E2 must penetrate fully into the interior of target MPO neurons to promote mating in castrated, DHT-treated male rats.

In order to test this hypothesis, in this study we compared the behavioral effects of delivering either E2 or E2 conjugated to bovine serum albumin (BSA-E2) to the MPO in castrated, DHT-maintained male rats. Bovine Serum Albumin (BSA) is a large protein that will not cross the plasma membrane and, when conjugated to steroids, can be used to isolate responses to steroids that are confined exclusively to the cell surface [117]. We
predicted that stimulation of MPO mER alone by BSA-E₂ would be insufficient to maintain mating behavior, whereas stimulation of all ER by E₂ would support normal mating.

**EXPERIMENTAL PROCEDURES**

*Animals.* Male and female Sprague-Dawley rats, at least 70 days of age, were obtained from Charles River Laboratories. Animals were housed in plastic cages, 22 x 44 x 18 cm, with free access to food and water in the university vivarium on a 14:10 hour reverse light:dark cycle (lights off at 0930 hours). Males were housed two per cage until the day of surgery; thereafter, they were singly housed. Females were housed two per cage throughout the study. All husbandry and surgical procedures were performed with permission of the IACUC and with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, revised 1985).

Female rats were anesthetized with isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an oxygen exchange rate of 500 cc per minute and transferring them to a nosecone dispensing 2-3% gas at 500 cc per minute oxygen exchange rate, ovariectomized, and a subcutaneous (s.c.) 6 mm Silastic (Dow Corning, Midland, MI) capsule (1.981 mm id X 3.175 mm od) filled with crystalline E₂ (Sigma, St. Louis, MO) was implanted in the scapular region. Thereafter, all females were allowed at least seven days to recover before the start of behavioral testing. On testing days, to induce estrus behavior, females received an injection of 1.0 mg progesterone in 0.2 ml sesame oil s.c. 4-6 hours before being paired with males.
Male rats were screened for sexual behavior in four weekly 30 minute mating
tests to determine their suitability for inclusion in the study. Based on total ejaculation
frequency, proven maters were assigned to three matched groups: (i) BSA-E₂ (N = 9, to
limit E₂ action to the cell surface), (ii) free E₂ (N = 7, to simulate ER in all cellular
locations) or (iii) vehicle (N = 8, inert control). Each male was anesthetized with
isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an
oxygen exchange rate of 500 cc per minute and transferring them to a nosecone
dispensing 2-3% gas at 500 cc per minute oxygen exchange rate, castrated and implanted
s.c. with a 10 mm Silastic capsule (1.981 mm id X 3.175 mm od) containing crystalline
DHT (Sigma). DHT capsules of this size have been reported to produce circulating DHT
levels in the physiological range [3, 82, 118]. Thereafter, they were stereotaxically
implanted bilaterally with 22 gauge, stainless steel, ethylene oxide sterilized, guide
cannulae (Plastics One, Roanoke, Virginia) aimed at the MPO (level skull coordinates: ap
= -0.5 mm, ml = ± 0.75 mm, dv = -8.0 mm [146]). Inner cannulae (28 gauge, Plastics
One) extended 1 mm below the guide cannulae and were tamped in either: (i) 1,3,5(10)-
estratrien-3, 17β-diol 17-hemisuccinate:BSA (Steraloids, Inc., Newport, Rhode Island,
code E1077-000; BSA-E₂), (ii) a 1:40 (wt/wt) mixture of unconjugated, crystalline E₂
(Sigma) plus unconjugated, crystalline BSA (Sigma; free-E₂) or (iii) a 1:40 (wt/wt)
mixture of unconjugated, crystalline cholesterol (Sigma) plus unconjugated, crystalline
BSA (vehicle), sterilized and inserted into the guide cannulae. Males were given five to
seven days to recover before the weekly postoperative behavioral testing began. Freshly
tamped sterilized inner cannulae were inserted into the brain 48 hours before and 24
hours after every behavioral test. In a group of pilot animals, daily microscopic
inspection of cannula tips for crystalline material ensured that this replacement schedule
would continuously expose the brain to the appropriate agent.

Sexual behavior tests. During preoperative sexual screening and postoperative
mating tests, each male was paired with a sexually receptive female for 30 minutes in a
22 x 44 x 50 cm testing arena under dim red light. Tests of this duration are sufficiently
long for male rats to obtain multiple ejaculations with females, and a 30 minute test
length is also used by other investigators [15]. Tests began 3 hours after lights went off
for the day. 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 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 neither occurred.

Histological verification. When the testing period was concluded, males were
euthanized with an overdose of sodium pentobarbital (100 mg/kg i.p.) and perfused
transcardially for 5 minutes 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 partially opened and immersed overnight in perfusion fixative. Brains were removed from skulls the next day and stored in 30% sucrose in 0.1 M PB for approximately 48 hours. Frozen coronal brain sections (40 μm) through the diencephalon were collected into 0.1 M PB, mounted on clean gel-albumin coated slides, and stained with Toluine blue to verify cannula placement.

**Estrogen receptor immunocytochemistry.** The H222 monoclonal anti-ER antibody (generous gift of Dr. Geoffrey Greene) was used at a concentration of 10 μl/ml. Coronal 40 μm MPO sections from animals in the three groups were processed concurrently with the same aliquots of antibodies and immunoreagents and were incubated first in a blocking solution containing 3% normal goat serum for 48 h at 4°C and second with the H222 anti-ER antibody for 48 h at 4°C. After three 5-min washes in 0.1 M PB, sections were then incubated for 2 h at room temperature with a biotinylated goat antirat secondary antibody (Jackson Laboratories, Bar Harbor, ME, code # 112-065-062, lot # 30138) at a dilution of 1:500. Sections were then washed in three 5-min changes of 0.1 M PB and incubated for 1 h at room temperature in an avidin-biotinylated peroxidase complex (Vector Elite Standard ABC kit, A and B reagents prepared at ½ the manufacture’s recommended concentration). Following three additional 5 min washes in 0.1 M PB, the sections were incubated for 10 min at room temperature in a solution containing 0.2 mg/ml diaminobenzidine (Sigma) plus 0.1 μl/ml 30% H₂O₂ (Sigma) dissolved in 0.1 M PB.

**Statistics.** Between-group behavioral data were analyzed by one-way or 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 [69]. Within-group comparisons of preoperative and postoperative changes in behavior were analyzed by paired t-tests [69]. Data from all males were used in the statistical analyses; default values (see sexual behavior tests) were used in situations where animals did not display a given behavior. Two-tailed probabilities are reported in all cases.

RESULTS

Male Sexual Behavior. Highly significant group differences emerged during the postoperative period. Animals receiving either BSA-E₂ or free E₂ in the MPO exhibited more robust mating behavior than vehicle controls on all behavioral indices and trials (FIGURE 6). Specifically, during postoperative trials, the groups differed significantly in mount frequency (group main effect: $F_{2,21} = 11.04, p < 0.001$), intromission frequency (group main effect: $F_{2,21} = 8.99, p < 0.002$), ejaculation frequency (group main effect: $F_{2,21} = 12.89, p < 0.001$), mount latency (group main effect: $F_{2,21} = 22.14, p < 0.001$), ejaculation latency (group main effect: $F_{2,21} = 15.17, p < 0.001$) and the post-ejaculatory interval (group main effect: $F_{2,21} = 16.57, p < 0.001$). Prior to surgery, however, the animals in all three groups had mated robustly and the groups did not differ significantly on any measure. Thus, all group differences manifested during the postoperative trials.

In a follow-up analysis, to compare the effects of E₂ versus vehicle, the two groups implanted in the MPO with either BSA-E₂ or free E₂ were pooled because post hoc comparisons from the analysis above indicated that they were statistically indistinguishable on every behavioral measure on all postoperative trials. The sexual
performance of this pooled estrogen group was subsequently compared with that of the vehicle controls. Both types of estrogen implants to the MPO promoted mating behavior, whereas vehicle implants did not maintain sexual behavior. Specifically, during postoperative trials, the pooled estrogen group performed significantly better than the vehicle group in mount frequency (group main effect: $F_{1,22} = 20.12, p < 0.001$), intromission frequency (group main effect: $F_{1,22} = 17.56, p < 0.001$), ejaculation frequency (group main effect: $F_{1,22} = 19.97, p < 0.001$), mount latency (group main effect: $F_{1,22} = 40.39, p < 0.001$), ejaculation latency (group main effect: $F_{1,22} = 26.27, p < 0.001$) and the post-ejaculatory interval (group main effect: $F_{1,22} = 32.41, p < 0.001$).

Subsequently, post hoc comparisons between the pooled estrogen and vehicle groups indicated significant increases ($p < 0.05$) in mount frequency on trials 2, 3 and 4; intromission frequency on trials 3 and 4; and ejaculation frequency on trials 2, 3 and 4; also, significant group decreases ($p < 0.05$) were observed in mount latency, ejaculation latency and the post-ejaculatory interval on all postoperative trials.

The behavior of each group was examined for changes during the preoperative and postoperative periods. In the BSA-E$_2$ group, for each dependent variable, there were no significant changes in mating behavior between the preoperative and postoperative periods. Specifically, no significant differences were noted between scores on the fourth preoperative test (representing terminal preoperative performance) and those on the last postoperative trial (representing terminal postoperative performance). Although mount frequency and intromission frequency scores in the free E$_2$ group were significantly lower on the fourth postoperative test compared to the fourth preoperative trial ($t = 3.47, df = 6$,
p < 0.01 and t = 4.17, df = 6, p < 0.005, respectively), most animals in this group nevertheless mated to ejaculation with females on both of these tests, and, with respect to ejaculation frequency, mount latency, ejaculation latency and the post-ejaculatory interval, scores did not differ significantly before and after surgery. In contrast, sexual performance in the vehicle control group deteriorated significantly after surgery. Specifically, mount frequency (t = 5.29, df = 7, p < 0.001), intromission frequency (t = 12.42, df = 7, p < 0.001) and ejaculation frequency (t = 4.71, df = 7, p < 0.002) decreased significantly between the last preoperative test and the fourth postoperative trial, and mount latency (t = 8.82, df = 7, p < 0.001), ejaculation latency (t = 4.91, df = 7, p < 0.002) and the post-ejaculatory interval (t = 4.61, df = 7, p < 0.002) increased significantly between the last preoperative test and the fourth postoperative trial.

**Cannula Placements.** Locations of cannula placements are indicated in FIGURE 7. Cannula tips were positioned in or near the medial preoptic area in all groups of animals.

**H222 Estrogen Receptor Immunoreactivity.** H222 ER ICC was performed to test the integrity *in vivo* of the bond between E2 and BSA. Neurons near the cannula tracts and ependymal zone of the MPO were positively labeled for H222 ER-ir in both the vehicle and BSA-E2 groups suggesting that intracellular ERs were not occupied by E2. Conversely, no H222 ER-ir was seen in the free E2 group indicating that E2 had penetrated the cell membrane to bind to intracellular ERs (FIGURE 8).
DISCUSSION

In this study we used MPO implants to compare the efficacy of free E2 and BSA-E2, a membrane-impenetrant form of E2, to support mating in castrated, DHT-maintained male rats. We found that BSA-E2 and free E2 were equally effective at maintaining copulatory behavior at significantly higher levels than did vehicle (which was ineffective in promoting mating behavior). This did not support our hypothesis that E2 must penetrate fully into the interior of target MPO neurons to promote mating in castrated, DHT-treated male rats. Instead, these findings show that E2 stimulation of mER is sufficient to promote mating and raise several questions regarding the mechanisms of E2 action, the nature of the receptor, the roles of intracellular ER, and the differential effects of E2 in various brain areas. Although the present data do not resolve all of these issues, the findings nevertheless support the ideas that stimulation of mER results in physiological [75, 138] and behavioral [42, 47] responses in vivo.

BSA-E2 and free E2 were equally effective in promoting mating (whereas the vehicle was ineffective) suggesting that stimulation of mER alone is sufficient for the display of male copulatory behavior. An earlier study [116] showed that blocking expression of ER-α in the MPO by chronic infusion of an antisense oligodeoxynucleotide (ODN) to ER-α mRNA attenuates mating. This is consistent with other reports suggesting that ER-α is the relevant ER for male mating behavior [123, 162]. Together, these findings imply that some form of ER-α operates as an mER, an idea that is further strengthened by Khan et al. [67] showing colocalization of ERα and MNAR/PELP1 (a scaffold protein and ER cofactor) at the plasma membrane of rat MPO neurons.
Although stimulation of ER-α in the MPO appears to be sufficient to induce mating behavior in DHT-maintained rats, it is interesting to note that this is not the case in the MEA. Blocking expression of ER-α in the MEA via antisense ODN infusion did not attenuate male mating behavior [116], suggesting that either ER-β or some novel form of ER, such as those described by Revankar and Thomas [128, 148], might mediate the actions of E2 in the MEA. Moreover, in contrast to this study, administration of BSA-E2 to the MEA of castrated, DHT-maintained males did not maintain mating behavior [60]. Thus, taken together, these results suggest differential actions of E2 in different brain areas, perhaps via alternative receptor mechanisms.

These interpretations depend on the stability of BSA-E2 in the brain. Published studies have successfully used BSA conjugated to steroids to investigate the rapid or membrane-limited signaling of hormones [22, 24, 47, 117, 161], and the use of such conjugates has become an accepted research method. Watters et al. [161] showed that BSA-E2 does not cleave while it promotes cellular responses \textit{in vitro} [180], although the action of an endogenous enzyme \textit{in vivo} cannot be ruled out. We inferred that cleavage of the bond between E2 and BSA does not occur in the rat brain because identical doses and aliquots of BSA-E2, when administered to the MEA, did not maintain mating [60]. As in the MPO, free E2 acts in the MEA to promote male sexual behavior [59] and, if cleavage of E2 from BSA-E2 had occurred, then rats with BSA-E2 MEA implants should have mated, but they did not. To confirm that E2 does not cleave from BSA-E2 \textit{in vivo}, in this study we used the H222 anti-ER antibody, which was raised in rat against full-length ER and recognizes the steroid-binding domain of human ER [55]. Previous studies
showed that the presence of E₂ interferes with H222 ER-ir labeling, whereas neurons are H222 ER-ir positive when E₂ is absent [6, 30]. Neurons were H222 ER-ir negative in MPO sections from the free E₂ group but were H222 ER-ir positive in the vehicle and BSA-E₂ groups, which argues that cleavage of the bond between E₂ and BSA had not occurred.

ER was initially described as a cytosolic protein that, upon binding E₂, translocated to the nucleus to induce mRNA transcription and protein translation. In 1997, a second ER was discovered in rat prostatic tissue [76], described as ER-β (the original ER is now referred to as ER-α). ER-β was also described as a nuclear transcription factor, although there are reports to the contrary [25, 176]. As early as 1977, Pietras suggested that an alternative ER associated with the cellular plasma membrane facilitated the rapid (seconds or minutes) effects of E₂ [121]. In recent years, reports of rapid effects of E₂, assumed to be mediated by mER, have increased substantially in number. Studies investigating these rapid cellular responses have raised complex issues that have yet to be resolved. For example, there is a consensus that these mERs mediate signal transduction events that, via kinase activation [24, 102, 161, 180], conformational changes in ion channels [66], or other mechanisms [21], lead to a rapid cascade of cellular responses, which, until recently, were thought of as non-genomic events. “Classical” action(s) of steroids has been defined as transcription events occurring in the nucleus and “non-genomic” action(s) of steroids has referred to rapid events that are presumed to be mediated by membrane-associated receptors. We now know that rapid effects of E₂ can also culminate in gene transcription [160] but do not
always do so [77]. Researchers must first address the confusions that arise from the use of terms such as “rapid”, “non-genomic” and “genomic” effects. Second, they must reach a consensus on the nature of the mER. Several reports suggest that alternative form(s) of ER-α may serve as the mER [34, 88, 155], but a small number of reports suggest that ER-β may function as an mER [25, 76]. Third, the manner in which mERs are associated with cell membranes is not fully understood. There are reports that mER has no extracellular domain [46, 142] or glycosylation [88], which suggests that mERs lie adjacent to the membrane but are not inserted into it. Conversely, some reports suggest that proteins couple ER to the plasma membrane [81]. Fourth, to further complicate matters, many reports have originated from in vitro studies of various tumor cell lines that do not normally express ER, but have been transfected to express them and in some cases these ER-negative cells (without endogenous ER) nevertheless respond to E2, suggesting either a non-receptor mechanism or an undescribed ER [109].

Our study does not address the question of whether E2 acts "classically" or "non-genomically" (or both) because our implants were chronic. It does show, however, that in the MPO, confining E2 exclusively to the cell surface maintains male rat mating behavior. Because cell surface actions of E2 can activate multiple signaling pathways, including genomic responses, more investigations are needed to determine the cellular mechanisms that influence mating. Nevertheless, these finding extend previous reports showing that rodents respond quickly to gonadal steroids by exhibiting mating-associated behaviors [42, 47] and also support the idea that steroids act differently in different brain areas [116]. Because a membrane impenetrant form of E2 maintained mating, we
conclude that a cell surface action of E$_2$ in the MPO is sufficient for expression of male sexual behavior.

**ACKNOWLEDGEMENTS**

This work partially fulfills the Doctor of Philosophy degree requirements of the Department of Biology, Georgia State University for G. G. Huddleston and was supported by National Science Foundation grant IBN 9876754 to the Center for Behavioral Neuroscience. We thank Dr. Geoffrey Greene for kindly donating the H222 anti-ER antibody, Dr. Laura Carruth for critically reviewing the manuscript and Ms. Donna Beauford for expert technical assistance.
Mating behaviors (mean ± standard error of the mean) after BSA-E2, BSA + E2 (free E2) or BSA + cholesterol (vehicle) implants to the MPO. Males implanted with vehicle to the MPO (squares) virtually ceased mating, whereas those implanted with either form of E2 (gray circles or triangles) mated robustly. Relative to vehicle controls, the pooled estrogen group showed significantly more mounts, intromissions and ejaculations and mount latency, ejaculation latency and the post ejaculatory interval were significantly shorter. Blackened squares indicate the trials where post hoc statistical comparisons revealed that the vehicle group differed significantly from the pooled estrogen group (p < 0.05, two-tailed).

* Significant within-group difference between the last postoperative trial and the last preoperative (PRE-) trial (p < 0.005, two-tailed).
FIGURE 1
HUDDLESTON ET AL., MPO MS

FIGURE 6
FIGURE 7

Locations of cannula tips in animals implanted with BSA-E₂ to the MPO (left), BSA + E₂ (free E₂; center) or BSA + cholesterol (vehicle; right). Cannula tips were positioned in or near the medial preoptic area.
FIGURE 8

H222 ER-ir immunoreactivity in the MPO. In MPO sections from the BSA-E2 group (left panel) and the vehicle group (right panel) neurons near the implant tracts and ependymal zone exhibited H222 ER-immunoreactivity but no labeled neurons were found in the free E2 group (center panel). Scale bar equals 25 μm.
FIGURE 8

BSA-E2 CONJUGATED

BSA + ESTRADIOL

BSA + CHOLESTEROL

FIGURE 3
HUDDLESTON ET AL., MPO MS
CHAPTER 3

EFFECTS OF ESTROGEN IN THE MALE RAT MEDIAL AMYGDALA:
INFUSION OF AN AROMATASE INHIBITOR LOWERS MATING AND
BOVINE SERUM ALBUMIN CONJUGATED-ESTRADIOL IMPLANTS DO
NOT PROMOTE MATING

Gloria G. Huddleston, Jacquelyn C. Paisley and Andrew N. Clancy, *Neuroendocrinology*,

ABSTRACT

In male rats, copulatory behavior depends on estrogen-responsive neurons located in brain areas known to be crucial for mating. Blocking the aromatization of testosterone (T) to estradiol (E₂) either throughout the brain or within the medial preoptic area (MPO) reduces mating, whereas E₂ treatment of either the MPO or the medial amygdala (MEA) maintains sexual behavior. The effects of T aromatization in the MEA have received less attention; therefore, two studies were done to further elucidate the effects of E₂ in the MEA. In Experiment 1, gonadally intact male rats that showed robust mating behavior were administered chronic Fadrozole, a non-steroidal aromatase inhibitor, to the MEA to stop the conversion of T to E₂ and then paired with receptive females. Infusion of Fadrozole to the MEA significantly lowered mating behavior in experimental males compared to vehicle-infused control males. To further investigate the mechanism by which E₂ acts in the MEA, in Experiment 2, E₂-conjugated to Bovine Serum Albumin (BSA-E₂: a complex of E₂ and a large protein that will not cross the plasma membrane, thereby restricting the action of E₂ to cell-surface signaling) was chronically administered bilaterally to the MEA of castrated, dihydrotestosterone-treated males. This treatment did not maintain mating behavior. These studies show E₂ acts in the MEA to promote male sexual behavior and suggest an intracellular mechanism of E₂ action.
INTRODUCTION

Mammalian male mating behavior depends on the actions of gonadal steroids on both central and peripheral structures. Testicular testosterone (T) is converted both peripherally and centrally to dihydrotestosterone (DHT) by 5-alpha reductase [90] and to estradiol (E₂) by aromatase [108]. Both metabolites, and T itself, interact to regulate male mating [151]. In female rats, receptivity (evidenced by lordosis) is mediated by an interconnected neuronal network composed of steroid-responsive (estrogen-responsive) cells [41, 119]. Presumably, a similar steroid-activated system or pathway governs the display of male copulatory behavior, an idea supported by the presence of estrogen receptors (ER) and/or androgen receptors (AR) colocalized with mating-induced Fos-ir in neurons of the medial preoptic area (MPO), the medial amygdala (MEA), the central tegmental field, the bed nucleus of the stria terminalis, and other areas known to mediate mating behavior [51].

The MPO is a brain area that is critical for the expression of male copulatory behavior. Electrical [28, 78] or chemical [56] lesions of the MPO attenuate male mating; and, conversely, electrical [87, 153] or chemical [43, 44] stimulation promotes it. We have previously shown the behavioral importance of E₂-responsive neurons of the MPO in studies using a non-steroidal aromatase enzyme inhibitor, Fadrozole, to block the conversion of T to E₂. Subcutaneous (s.c.) administration of Fadrozole significantly decreased the conversion of [³H]T to [³H]E₂ in male rat hypothalamic and amygdaloid nuclear fractions [12]. Moreover, administering Fadrozole s.c. (but not vehicle) to castrated, T-maintained male rats significantly decreased mating behavior, and
subsequent administration of E$_2$ partially restored some copulatory behaviors [12].

Chronic intracranial infusion of Fadrozole (but not saline) bilaterally to the MPO also significantly decreased mating [32]. Conversely, mating behavior was maintained in rats given chronic Fadrozole s.c. (to block T aromatization throughout the brain) together with bilateral MPO implants of E$_2$ (but not vehicle) [33]. Anatomical controls showed that Fadrozole is not toxic to the brain and does not interfere with behaviors, with the exception of those that are dependent on the estrogenic metabolites of T [32].

The MEA, a limbic site that is reciprocally connected to the MPO [18, 41, 136], receives chemosensory [37], genital-somatosensory [8, 52] and other projections necessary for male sexual behavior and also contains ER [51]. Because mating-induced Fos-ir is colocalized with ER in neurons of the MEA, the same experimental approach (chronic Fadrozole s.c. plus bilateral intracranial E$_2$ implants) that proved useful in our previous investigation on the effect of E$_2$ in the MPO was used to determine the effects of E$_2$ in the MEA. Male rats receiving Fadrozole s.c. and vehicle implants to the MEA showed significant reductions in mounting, intromission and ejaculation; however, males receiving Fadrozole s.c. and bilateral E$_2$ implants to the MEA mounted and intromitted at levels that were not significantly different from presurgical levels, although ejaculation was not maintained [59]. These findings suggest that estrogen-responsive MEA neurons contribute to male sexual behavior. To test this hypothesis, we performed, in Experiment 1, the opposite manipulation: Fadrozole was chronically administered bilaterally to the MEA of sexually experienced, gonadally intact male rats. We predicted this would deprive the MEA of E$_2$ aromatized from T and significantly lower male mating behavior.
The mechanism(s) by which E$_2$ exerts its effects on neurons is poorly understood. Traditionally, it had been thought that E$_2$ functioned only as a selective transcription regulator of DNA [64]. We now know that there is a diversity of ERs in various cellular locations. ERs have been described in the nucleus [68, 76], the cytoplasm [10], tethered to intracellular membranes [100, 101, 128, 176], and both classical [25, 121, 122, 155] and novel [128, 148] forms of ER have been described as being associated with the plasma membrane. Non-nuclear ERs are believed to mediate rapid effects of E$_2$ (seconds to minutes) by activating cellular signaling cascades that might or might not culminate in gene transcription. This raises questions as to whether E$_2$ acts at the cell surface, intracellularly or both. Previous studies have used either progesterone [47] or E$_2$ [117] conjugated to Bovine Serum Albumin (BSA), a large protein that does not cross the plasma membrane [125], to limit the effects of hormone to the membrane. We hypothesized that E$_2$ would act at the plasma membrane in the MEA to maintain mating behavior. Therefore, in Experiment 2, E$_2$ conjugated to BSA (BSA-E$_2$) was chronically administered bilaterally to the MEA of sexually experienced, castrated, DHT-maintained male rats, which we anticipated would maintain mating.

**MATERIALS AND METHODS**

*Animals.* Male and female Sprague-Dawley rats, at least 70 days of age, were obtained from Charles River Laboratories. Animals were housed in plastic cages, 22 x 44 x 18 cm, with free access to food and water in the university vivarium on a 14:10 hour reverse light:dark cycle (lights off at 0930 hours). Males were housed two per cage until
the day of surgery; thereafter, they were single housed. 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).

Female rats were anesthetized with isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an oxygen exchange rate of 500 cc per minute and transferring them to a nosecone dispensing 2-3% gas at 500 cc per minute oxygen exchange rate, ovariectomized and given s.c. a 6 mm Silastic capsule (1.981 mm id X 3.175 mm od) filled with crystalline E2. Thereafter, all females were allowed at least seven days to recover before the start of behavioral testing. On testing days, females received 1.0 mg progesterone in 0.2 ml sesame oil s.c. 4-6 hours before being paired with males.

Male rats were screened for sexual behavior in four weekly 30 minute mating tests to determine their suitability for inclusion in the study; and in each experiment, groups of proven maters were matched based on total ejaculation frequency. In the first study, males were assigned to one of two groups: Fadrozole-in-MEA or Saline-in-MEA. In the second study, males were assigned to one of two groups: BSA-E2-in-MEA or cholesterol-in-MEA.

**Experiment 1: Fadrozole and saline administration to MEA.** Each male was anesthetized with isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an oxygen exchange rate of 500 cc per minute and transferring them to a nosecone dispensing 2-3% gas at 500 cc per minute oxygen exchange rate and
stereotaxically implanted bilaterally with 25 or 28 gauge stainless steel, ethylene oxide sterilized cannulae (Plastics One, Roanoke, Virginia) aimed at the MEA (level skull coordinates: ap = -3.2 mm, ml = ± 3.5 mm, dv = -9.2 mm [146]) and connected via tubing to an osmotic minipump (ALZET models 2002 or 2004). In the saline group (n = 11), cannulae delivered 6 μl sterile isotonic saline per 24 hrs to each side of the brain. In the Fadrozole group (n = 9), cannulae delivered 0.80 - 1.38 μg Fadrozole (CGS-16949A, CIBA-Geigy Corporation, Summit, NJ; doses varied according to the minipump model used) in 6 μl saline per 24 hrs to each side of the brain. Intracranial Fadrozole doses in this range have been reported to block the local conversion of E₂ from T [30] and have been used successfully by other investigators [32, 151]. Prior to surgery, minipumps were incubated at 37° C for 48 hours in physiological saline to ensure full pumping activity at the time of brain implantation. Males were given five to seven days to recover before weekly postoperative behavioral testing began. Model 2002 minipumps (which dispense for 14 days) were replaced after 13 days but model 2004 minipumps (which dispense for 28 days) were not removed until the end of the study. Fluid levels in all pumps were checked to determine that there had been no pump failures; none occurred.

**Experiment 2: BSA-E₂ and cholesterol administration to males.** Each male was anesthetized with isoflurane gas by placing them in a chamber with an atmosphere of 4% gas and an oxygen exchange rate of 500 cc per minute and transferring them to a nosecone dispensing 2-3% gas at 500 cc per minute oxygen exchange rate, castrated and implanted s.c. with a 10 mm Silastic capsule (1.981 mm id X 3.175 mm od) containing crystalline DHT (Sigma). DHT capsules of this size have been reported to produce
circulating DHT levels in the physiological range [3, 82, 118] and were behaviorally effective in another study in which we used identical DHT capsules s.c. and found that mating occurred if male rats were given either E2 or BSA-E2 implants to the MPO, whereas DHT-treated males with vehicle MPO implants did not mate [61]. Thereafter, they were stereotaxically implanted bilaterally with 22 gauge stainless steel, ethylene oxide sterilized guide cannulae (Plastics One) aimed at the MEA (level skull coordinates: ap = -3.2 mm, ml = ± 3.5 mm, dv = -8.2 mm [146]). Inner cannulae (28 gauge, Plastics One) extended 1 mm below the guide cannulae and were tamped in either: (i) 1,3,5(10)-estratrien-3, 17β-diol 17-hemisuccinate:BSA (Steraloids, Inc., Newport, Rhode Island, code E1077-000; BSA-E2 group) or (ii) a 1:40 (wt/wt) mixture of unconjugated, crystalline cholesterol (Sigma) plus unconjugated, crystalline BSA (cholesterol group), sterilized and inserted into the guide cannulae. Males were given five to seven days to recover before the weekly postoperative behavioral testing began. Freshly tamped, sterilized inner cannulae were inserted into the brain 48 hours before and 24 hours after every behavioral test. In a group of pilot animals, daily microscopic inspection of cannula tips for crystalline material ensured that this replacement schedule would continuously expose the brain to the appropriate agent. In addition, when cannulae from experimental animals were inspected, crystalline material was present at the cannulae tips at least 85% of the time. There were 7 animals in the BSA-E2 group and 8 animals in the cholesterol group.

**Sexual behavior tests.** During preoperative sexual screening and postoperative mating tests, each male was paired with a sexually receptive female for 30 minutes in a
22 x 44 x 50 cm testing arena under dim red light. Tests of this duration are sufficiently long for male rats to obtain multiple ejaculations with females, and a 30 minute test length is also used by other investigators [15]. Tests began 3 hours 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 or a default of 1800 sec if no mounts occurred; ejaculation latency (EL) - time from the first mount or intromission until the first ejaculation 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 neither occurred.

**Histological verification.** When the testing period was concluded, males were euthanized with an overdose of sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially for 5 minutes 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 partially opened and immersed overnight in perfusion fixative. Brains were removed from skulls the next day and stored in 30% sucrose in 0.1 M PB for approximately 48 hours. Frozen coronal brain sections (40 μm) through the diencephalon were collected into 0.1 M PB, mounted on gel-albumin coated slides and stained with Toluine blue to verify cannula placement.
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; and within-group comparisons of preoperative and postoperative changes in behavior were analyzed by paired t-tests [69]. Data from all males was used in the statistical analyses; default values (see sexual behavior tests) were used in situations where animals did not display a given behavior. Two-tailed probabilities are reported in all cases.

RESULTS

Experiment 1

Male Sexual Behavior: Relative to saline-infused controls, which displayed robust mating behavior postoperatively, animals infused with Fadrozole to the MEA demonstrated statistically significant reductions in intromission frequency (group main effect: F1,18 = 9.08, p < 0.007, group x trial interaction: F3,54 = 2.89, p < 0.043) and ejaculation frequency (group main effect: F1,18 = 6.26, p < 0.021), whereas mount latency (group main effect: F1,18 = 8.13, p < 0.01, group x trial interaction: F3,54 = 4.29, p < 0.009), ejaculation latency (group main effect: F1,18 = 5.55, p < 0.028) and the post-ejaculatory interval (group main effect: F1,18 = 12.97, p < 0.002) were significantly increased. Mount frequency did not differ significantly between the two groups (FIGURE 9 and TABLE 1). Follow-up post hoc statistical comparisons indicated that intromission frequency was significantly reduced (p < 0.05) in Fadrozole-infused males relative to saline-infused controls on the fourth postoperative week, whereas ejaculation
frequency was significantly depressed (p < 0.05) on the third postoperative week. Similarly, Fadrozole-infused animals had significantly longer (p < 0.05) mount latencies on the fourth postoperative week, significantly greater (p < 0.05) ejaculation latencies on the third postoperative week and significantly larger (p < 0.05) post-ejaculatory interval scores on the third and fourth postoperative weeks. In saline controls, for each dependent variable, there were no significant differences between performance on the last week immediately preceding surgery (representing terminal preoperative performance) and the last postoperative week (representing terminal postoperative performance). No significant differences were noted between groups on any behavioral measure during preoperative weeks. In the Fadrozole group, however, comparisons between last preoperative week and the last postoperative test indicated there was a significant decline in mount frequency (t = 4.21, df = 8, p < 0.003) and intromission frequency (t = 2.81, df = 8, p < 0.02) and a significant increase in mount latency (t = 4.13, df = 8, p < 0.003) after surgery.

**Cannula Placements:** Locations of cannula placements are indicated in FIGURE 10. Cannula tips were positioned in or near the medial amygdala in both groups of animals.

**Experiment 2**

**Male Sexual Behavior:** Vehicle-implanted controls demonstrated modest, but significantly lower, intromission frequency (group main effect: F1,13 = 5.02, p < 0.041) and ejaculation frequency (group main effect: F1,13 = 6.00, p < 0.028) than did animals
given BSA-E₂ to the MEA, and their post-ejaculatory interval (group main effect: \( F_{1,13} = 7.72, p < 0.015 \)) was significantly longer. Follow-up post hoc comparisons revealed no other significant group differences (FIGURE 11 and TABLE 2). Each group exhibited robust mating prior to surgery and did not differ significantly on any behavioral measure. Postoperatively, each group showed a significant decline in mating behavior. In the group implanted with BSA-E₂ to the MEA, comparisons of the performance on the last preoperative week and the last postoperative week indicated that mount frequency (\( t = 2.89, df = 6, p < 0.028 \)), intromission frequency (\( t = 4.41, df = 6, p < 0.018 \)) and ejaculation frequency (\( t = 3.05, df = 6, p < 0.022 \)) were significantly reduced postoperatively. Moreover, in the vehicle-implanted group, comparisons between the preoperative last week and the last postoperative test revealed that mount frequency (\( t = 3.01, df = 7, p < 0.02 \)), intromission frequency (\( t = 5.23, df = 7, p < 0.0012 \)) and ejaculation frequency (\( t = 2.99, df = 7, p < 0.02 \)) were significantly lower postoperatively; and mount latency (\( t = 2.54, df = 7, p < 0.039 \)), ejaculation latency (\( t = 3.51, df = 7, p < 0.0099 \)) and the post-ejaculatory interval (\( t = 3.81, df = 7, p < 0.0067 \)) were significantly increased.

**Cannula Placements:** Locations of cannula placements are indicated in FIGURE 12. Cannula tips were positioned in or near the medial amygdala in both groups of animals.
**DISCUSSION**

These studies had two goals: (i) to demonstrate the necessity of E$_2$ in the MEA for the expression of male sexual behavior and (ii) to begin to investigate the mechanism(s) by which E$_2$ mediates the behavior. The first study showed that inhibiting the aromatization of T to E$_2$ in the MEA resulted in significant reduction of male sexual behavior, supporting the hypothesis that E$_2$ acts in the MEA to promote male mating behavior. This extends previous reports that E$_2$ implants to the MEA mediate sexual behavior [59]. The second study showed that BSA-E$_2$ implants to the MEA did not support mating, counter to the hypothesis that E$_2$ would act at the plasma membrane in the MEA to maintain mating behavior.

Our findings from the first experiment support the idea that estrogenic metabolites of T act in the MEA to facilitate the expression of male rat mating behavior. Administration of E$_2$ to the cortical amygdala of castrated male rats restored mounting behavior [126], and E$_2$ administered to the MEA of castrated male hamsters increased mounting and other non-couplatory responses associated with mating behavior [167]. In another study with gonadally intact rats, systemic Fadrozole administration reduced mating, but mounting and intromission were maintained by E$_2$ (but not vehicle) implants to the MEA of Fadrozole-treated males [59]. In the present study with gonadally intact rats, we showed that blocking the formation of E$_2$ from T in the MEA via chronic infusion of Fadrozole reduced all indices of sexual behavior but did not entirely eliminate mating. Together, these findings suggest that E$_2$ acts within the male rat MEA to promote mating behavior.
Blocking the formation of E\textsubscript{2} in the MPO also attenuates mating behavior [32]. Specifically, gonadally intact males receiving chronic infusion of Fadrozole to the MPO displayed severe deficits in mounting, which ultimately resulted in significantly suppressed ejaculation. Conversely, all components of copulatory behavior were maintained, albeit at somewhat low levels, in males given Fadrozole s.c. and MPO E\textsubscript{2} implants [33]. Thus, converging evidence suggests that E\textsubscript{2} acts within the MPO, the MEA and probably other brain areas where populations of E\textsubscript{2}-responsive neurons mediate copulatory performance. While it is likely that both T and/or its androgenic metabolites [151] are also required for full, robust behavior to occur, it is clear that E\textsubscript{2} is necessary in these brain areas, presumably acting through ER [116, 151, 162], although questions remain regarding the mechanism(s) by which E\textsubscript{2} exerts its behavioral effects.

Traditionally, it had been believed that E\textsubscript{2} facilitates mating by acting genomically after binding to its intracellular receptors, translocating to the nucleus and interacting with DNA to induce mRNA transcription and protein synthesis [64, 68, 93, 164]. Presumably, these E\textsubscript{2}-dependent proteins perform functions that might be conducive to the expression of certain behaviors. It is possible these induced proteins are enzymes that enable the neurons to manufacture specific neurotransmitters that are required for mating. For example, E\textsubscript{2} induces changes in levels of cholecystokinin (CCK) in brain areas that mediate female rat sexual behavior [99, 113, 137]. Moreover, T modulation of CCK has been reported to occur in areas of the male rat brain that are important for the display of copulatory behavior, and it is possible that these changes result from the aromatization of T to E\textsubscript{2} [135]. These studies raise the possibility that E\textsubscript{2} acts as a “chemical switch” [137].
regulating differential levels of neurotransmitters in behaviorally-relevant neurons. Such slow genomic actions have been referred to as “classical” actions of E2; however, recently there have been reports of more rapid effects of E2. These fast responses to E2 are believed to be cell-signaling effects coupled to G protein or other pathways that induce, for example, intracellular calcium signals [21], phosphorylation [13], or kinase signaling [124, 139]; and they are presumed to be mediated by ERs associated with the plasma membrane, the intracellular membranes [128, 176], or the cytosol whose actions are at sites other than the nucleus [1, 139, 179].

To further complicate attempts to define the role of E2 in behavior, there are two known “classical” receptor types, ER-alpha and ER-beta, and both types are found not only in the nucleus [49, 68, 76, 132] but also in the cytosol [10, 11, 79, 100, 101, 179] and can be inserted into the plasma membranes [127]. Because of the well documented role that E2 plays in the MPO and the MEA in the expression of male mating, and because so little is known about the mechanism(s) of its action, we investigated the behavioral effects of blocking the synthesis of ER-alpha by chronically infusing antisense oligodeoxynucleotides (ODN) complementary to the start translation site of ER-alpha mRNA into the MPO and MEA. Chronic infusion of ODN to the MPO attenuated mating, but ODN infusion to the MEA had no discernable effects on copulation, suggesting that E2 is acting differently in these two brain areas [116]. These findings suggest that ER-alpha is the behaviorally-relevant ER in the MPO but not the MEA. This raises questions such as: Which ER is behaviorally relevant in the MEA? and How does E2 exert its differential effects in the MPO and MEA? It is possible that ER-beta is
behaviorally relevant in the MEA; however, studies in mice do not support this interpretation [162]. Another possibility is that a plasma membrane-associated ER of classical or novel form mediates the response to E2 in the MEA. We used a membrane-impenetrant form of E2 to test the latter possibility.

Previous studies have successfully shown that conjugation of a steroid to BSA, a large protein that does not cross the plasma membrane, can demonstrate the rapid effects of membrane-associated receptors [47, 117, 125]. We therefore tested the efficacy of BSA-E2 administered to the MEA of castrated, DHT-treated male rats to maintain mating behavior. Chronic delivery of BSA-E2 to the MEA did not maintain mating behavior and did not support our hypothesis, that E2 mediates sexual behavior by acting at the plasma membrane of E2-responsive MEA neurons. Instead, BSA-E2-treated males exhibited significant declines in mating behavior compared to their preoperative levels. Moreover, BSA-E2-treated males performed only slightly better than vehicle controls on a few indices of mating behavior. Although we did not assay for the cleaved products in this experiment, it is unlikely there was cleavage of the bond between the BSA molecule and the hormone. In a companion study in which BSA-E2 was administered to the MPO, immunocytochemistry using the H222 anti-ER antibody (which recognizes unoccupied ER) produced labeling, demonstrating that cleavage did not occur [61]. Furthermore, had cleavage occurred, E2 would have been free to enter MEA neurons and promote mating, as did the administration of E2 to the MEA in a previous study [59]. It is also unlikely that the concentration of E2 in the BSA-E2 was too dilute to support mating because the
dose used here was the same dose that stimulated mating when administered to the MPO [61].

Blocking the synthesis of ER-alpha in the MEA did not attenuate sexual behavior, but the same treatment in the MPO significantly reduced mating [116]. Chronic administration of BSA-E2 to the MPO also maintained mating behavior [61], raising the possibility that the behaviorally-relevant ER (alpha) is associated with the plasma membrane of neurons in the MPO as suggested by Khan et al. [67]. This is not the case in the MEA, where stimulation of plasma membrane-associated receptors alone (if present in the MEA) via the use of BSA-E2 did not support mating. Some investigators speculate that hormonal effects in the brain are transduced into behaviors when rapid membrane-mediated events act in concert with traditional genomic or classical models of hormone activity in the nucleus [154]. Whether or not this model is valid cannot be addressed by the data presented here. Nevertheless, the Fadrozole study extends and validates the ideas that E2 acts in the male rat MEA to promote mating and that the local conversion of T to E2 is necessary for the expression of copulatory behavior. We interpret the outcome of the BSA-E2 study as a guidepost indicating how E2 may act in the MEA. We propose that E2 acts intracellularly within the MEA rather than at the plasma membrane, perhaps via a novel mechanism, to facilitate male rat copulatory behavior.
ACKNOWLEDGEMENTS

This work partially fulfills the Doctor of Philosophy degree requirements of the Department of Biology, Georgia State University for G. G. Huddleston and was supported by Whitehall Foundation grant J96-24 and National Science Foundation grant IBN 9876754 to the Center for Behavioral Neuroscience. We thank Dr. Laura Carruth for critically reviewing the manuscript and Ms. Donna Beauford for expert technical assistance.
FIGURE 9

Mating behavior (mean ± standard error of the mean, SEM) after Fadrozole or saline infusion of the MEA. Males infused with saline to the MEA (gray squares) mated robustly, whereas those infused with Fadrozole to the MEA (circles) mated poorly, showing significantly fewer intromissions and ejaculations than saline controls. Relative to saline controls, Fadrozole-infused animals showed significant increases in mount latency, ejaculation latency and the post ejaculatory interval. Blackened circles indicate the weeks where post hoc statistical comparisons revealed that the Fadrozole group differed significantly from the saline group (p < 0.05, two-tailed).

* Significant within-group difference between the last postoperative week and the last preoperative (PRE-) week (p < 0.02, two-tailed).
FIGURE 9

FADROZOLE (N = 9): p = n.s. & p < 0.05
SALINE (N = 11):

FIGURE 1
HUDDLESTON ET AL., MEA MS
FIGURE 10

Locations of cannula tips of animals infused with Fadrozole to the MEA (left) and those infused with saline (right). Cannula tips were positioned in or near the medial amygdala.
FIGURE 2
HUDDLESTON ET AL., MEA MS

FIGURE 10
FIGURE 11

Mating behavior (mean ± SEM) after BSA-E₂ or vehicle implants to the MEA. Mating behavior was poor in both groups, although males implanted with BSA-E₂ to the MEA (circles) showed slightly but significantly more intromissions and ejaculations compared to vehicle-implanted controls (gray squares), and the post ejaculatory interval was significantly shorter. Post hoc statistical comparisons revealed there were no weeks where the BSA-E₂ group differed significantly from the vehicle group.

* Significant within-group difference between the last postoperative week and the last PRE- week (p < 0.04, two-tailed).
FIGURE 3
HUNDELESTON ET AL., MEA MS
FIGURE 12

Locations of implant tips of animals in the BSA-E₂ conjugated group (left) and the BSA + cholesterol group (right). Implants were located in or near the medial amygdala.
TOP

BSA-E₂ CONJUGATED

BSA + CHOLESTEROL

FIGURE 4
HUDDLESTON ET AL., MEA MS

FIGURE 12
<table>
<thead>
<tr>
<th>BEHAVIOR:</th>
<th>GROUP:</th>
<th>WEEK:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-operative Week</td>
</tr>
<tr>
<td>Mount Latency</td>
<td>Fadrozole</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>100%</td>
</tr>
<tr>
<td>Ejaculation Latency</td>
<td>Fadrozole</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>100%</td>
</tr>
<tr>
<td>Post-ejaculatory interval</td>
<td>Fadrozole</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>100%</td>
</tr>
<tr>
<td>BEHAVIOR:</td>
<td>GROUP:</td>
<td>WEEK:</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-operative Week</td>
</tr>
<tr>
<td>Mount Latency</td>
<td>BSA-E2</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>88%</td>
</tr>
<tr>
<td>Ejaculation Latency</td>
<td>BSA-E2</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>75%</td>
</tr>
<tr>
<td>Post-ejaculatory interval</td>
<td>BSA-E2</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>75%</td>
</tr>
</tbody>
</table>
CHAPTER 4

COLOCALIZATION OF STEROID HORMONE RECEPTORS AND PSEUDORABIES VIRUS FOLLOWING INJECTION INTO THE PROSTATE GLAND OF MALE RATS

ABSTRACT

Mating-induced Fos-immunoreactivity (ir) is colocalized with androgen Receptors (AR) or estrogen receptors (ER) or both in limbic and hypothalamic areas that have been shown to mediate the display of male rat mating behavior. A steroid-responsive neural network might govern copulatory behavior in males that is similar to the network described that governs the lordotic response in female rats. It is probable that this hypothesized network synchronizes and coordinates sexual behavioral responses with physiological responses of the genitals and the internal reproductive organs associated with successful reproduction. An attenuated form of the Bartha’s K strain of Pseudorabies Virus (PRV), a trans-synaptic retrograde tracer, was injected into the prostate gland (14 μl of 3 x 10⁸ pfu). After seven days, brains from infected animals were processed and double labeled for AR-ir + PRV-ir and ER-ir + PRV-ir. The majority of PRV-ir neurons expressed either AR-ir or ER-ir the medial preoptic areas (MPO), the medial preoptic nucleus (MPN), the bed nucleus of the stria terminalis (BST), the paraventricular nucleus hypothalamus (PVH), and the zona incerta (ZI): areas that have been shown to play a role in male mating. Areas such as the periaqueductual gray (PAG) and the central nucleus of the amygdala (CEA) and the medial amygdala (MEA) that have also been shown to be instrumental in the display of male copulatory behavior were less reliably labeled. Nevertheless, we described a steroid-responsive, neuronal circuit in the diencephalon that might initiate the motor aspect of mating and synchronize autonomic and behavioral sexual responses.
INTRODUCTION

In female rodents a steroid-responsive brain network has been described that mediates lordosis, the female copulatory reflex [41, 119]. Because such a network exists in females, and because gonadal hormones must act on hypothalamic and limbic sites of the male rodent brain for sexual behavior to occur, it is reasonable to hypothesize that a similar brain neural network, composed of interconnected, steroid-sensitive neurons, mediates male mating behaviors. Historically, lesion and stimulation studies have suggested that hypothalamic and limbic areas including, but not limited to, the medial preoptic area (MPO), the medial amygdala (MEA) and the bed nucleus of stria terminalis (BST) mediate male sexual behaviors [63, 97] and anatomical studies have shown that these areas are reciprocally interconnected [18, 38, 39, 136]. Moreover, neurons in each of these brain regions contain steroid hormone receptors, either androgen receptors (AR) alone, estrogen receptors (ER) alone, or both receptor types [51, 132]. Male sexual behavior is altered or attenuated when steroid hormone receptor antagonists to AR or ER are administered to the MPO or the MEA (and probably to the BST) [92, 94, 95, 152]. Hormone manipulation studies using a variety of techniques, including inactivation of the aromatase enzyme that converts T to E₂ [108], reinforce the idea that the display of male copulatory behavior is dependent on gonadal hormone action on the brain and suggest that the role of estrogen in male sexual behavior may be greater than previously recognized [12, 33, 59, 60, 116, 130]. Immunocytochemical (ICC) studies using Fos immunoreactivity (ir) as a marker of neuronal activity showed that mating-induced Fos-ir is colocalized with either ER-ir or AR-ir or both in these same brain areas [51]. Thus, it
is likely that the interconnected brain regions that make up the hypothesized steroid-sensitive network synchronize and coordinate the sexual behavioral responses with the physiological responses of the peripheral genital apparatus and the internal reproductive organs associated with insemination.

To test the hypothesis we injected an attenuated form of the pseudorabies virus (PRV), the Bartha’s K strain, a transneuronal, retrograde tract tracer, into a peripheral structure that is activated during mating, the prostate gland. When this viral strain is introduced peripherally, it is taken up by autonomic motor neuron terminals [144]. Inside the infected neuron, the virus self-replicates and is transferred across synapses in a retrograde fashion to presynaptic axon terminals. The process is repeated and viral infection spreads from neuron-to-neuron in a chain-like fashion [19, 20] such that, with anti-PRV ICC, an entire multisynaptic neural network can be visualized, one that presumably controls the functions of the peripheral structure. We elected to infect the prostate gland with PRV because the expulsion of prostatic fluid is temporally synchronized with the ejaculatory behavioral pattern, which suggests that activity in a common, supraspinal circuit may trigger both the expulsion of prostatic fluid and the behavioral patterns associated with mating behavior. We reasoned that PRV would infect the central neural network underlying mating behavior and we predicted that this network would be composed of steroid-sensitive neurons. Brains were dually processed for the expression of PRV-ir and either ER-ir or AR-ir and we anticipated finding a high proportion of dual-labeled neurons in brain areas known to mediate male rat sexual behavior.
MATERIALS AND METHODS

Animals

Male (371-605g) Sprague-Dawley rats, at least 70 days of age, were obtained from Charles River Laboratories. Animals were housed singly in polycarbonate cages, 22 x 44 x 18 cm, with free access to food and water in the university vivarium on a 14:10 hour reversed light:dark cycle (lights off at 0930 hours). All housing and experimental procedures were approved by the Institutional Animal Care facility and were in accordance with PHS guidelines. Males were anesthetized to a surgical plane with sodium pentobarbital (40 mg/kg i.p., Veterinary Laboratories, Inc., Lenexa, Kansas) and the prostate gland was exposed via a small ventral incision directly over the bladder. The prostate was supported by a sterile probe and the PRV was microinjected into the prostate glands of male rats using a Hamilton Syringe, as described in detail below.

PRV Injection and dissection

Each animal (N = 20) received a total of 14 μl PRV (the virus titer was 3 x 10^8 plaque forming units per ml), delivered as follows: 7 μl PRV was distributed into each of two prostatic lobes by microinjecting 1 μl followed by holding the syringe in place for one minute, then slightly moving the syringe to inject the next μl at a different sites in the lobe. Animals were monitored daily for signs of infection or urinary discomfort. After seven days, animals were given a lethal dose (100-150 mg/kg) of pentobarbital and perfused transcardially with physiological saline followed by a minimum of 300 ml of 4% paraformaldehyde. Skulls were partially opened and immersed in the perfusion fixative. The next day, brains were removed from skulls and transferred to 30% sucrose
in 0.1 M sodium phosphate buffer (PB) (pH 7.4) for three days. Brains were immersed in 0.1 M PB overnight, cut coronally into 40 μm sections with a freezing microtome, and then stored in 0.1 M PB. Immunocytochemistry was used (see below) to visualize infected neurons.

**Immunocytochemistry**

**Dual Labeling for ER-ir and PRV-ir**

Sections were rinsed in fresh 0.1 M PB followed by a 24 h incubation in a blocking solution of 0.1 M PB + 0.4% Triton X (TX) + 5% normal goat serum (NGS) (Jackson Laboratories, Bar Harbor, ME) at room temperature with agitation. Sections were then sequentially incubated with two different primary antibodies: first, with the RB132 rabbit polyclonal anti-PRV antibody (gift of Dr. L Enquist, Princeton University, dilution = 1:10,000) in a solvent of 2% NGS and 0.1% sodium azide in 0.1 M PB + 0.4% TX for 24 h at room temperature with agitation, and second, with the 1D5 mouse monoclonal anti-ER antibody (Zymed Laboratories, South San Francisco, catalogue # 18-7149 concentration = 1 μg/ml) in a mixture of 2% NGS in 0.1 M PB + 0.4% TX for 48 h at room temperature with agitation. After three 5 min washes in 0.1 M PB, sections were incubated in a mixture of two secondary antibodies: (i) biotinylated goat anti-rabbit secondary antibody (1:350 dilution, Vector Laboratories, Burlingame, CA catalogue # BA-1000, Lot No. A0708) and (ii) gold-conjugated goat anti-mouse secondary antibody (1:200 dilution, Jackson Laboratories, catalogue # 115-185-100, Lot No. 44811) in a solution of 0.1 M PB + 0.4% TX + 3% NGS for 2 h at room temperature with agitation. For the peroxidase reaction, sections were washed three times for 5 min in 0.1 M PB,
incubated in one-tenth-strength avidin-biotinylated peroxidase complex (Vector Laboratories, Vectastain Elite standard ABC peroxidase kit) in 0.1 M PB for 60 min at room temperature with agitation, washed in three times for 5 min in 0.1 M PB, and then incubated in a mixture of diaminobenzidine (DAB, Sigma, St. Louis, MO concentration = 0.2 mg/ml) and H₂O₂ (Sigma, concentration = 0.1 μl/ml) in 0.1 M PB for 5 min at room temperature. For the silver-enhancement reaction, sections were washed three times for 5 min in deionized water, incubated in silver-enhancement reagents (BBI Silver Enhancement kit, Ted Pella, Redding, CA prepared according to the manufacturer’s directions) for 20 min at 4°C and then washed three more times in deionized water for 5 min each. Finally, after three additional 5 min washes in 0.1 M PB, sections were mounted, dehydrated in alcohol, cleared with xylene, and cover-slipped with DPX mountant. Positive control sections from previously verified PRV infected animals were included in each assay for assay reliability.

**Dual Labeling for AR-ir and PRV-ir**

Sections adjacent to those used in the ER-ir + PRV-ir ICC assay were dually processed for AR-ir and PRV-ir as described above except that the incubation in the NGS blocking solution was at 4°C, the silver-enhancement reaction was conducted at room temperature, and different primary and secondary antibodies were used. At the appropriate steps in the procedure, sections were sequentially incubated with two different primary antibodies: first, with a porcine polyclonal anti-PRV antibody (gift of Dr. L Enquist, dilution = 1:30,000) in a solvent of 5% NGS in 0.1 M PB + 0.4% TX, for 24 h at 4°C, and second, with the PG-21 rabbit polyclonal anti-AR antibody (Upstate,
Cat. No. 06-680, Lot No. 24732, concentration = 3 μg/ml) in the same solvent for 48 h at 4°C. After three 5 min washes in 0.1 M PB, sections were incubated in a mixture of two secondary antibodies: (i) biotinylated goat anti-swine secondary antibody (1:200 dilution, Vector BA-9020, Lot No. K0223) and (ii) gold-conjugated goat anti-rabbit secondary antibody (1:200 dilution, BBI LM.GAR5, Batch No. 2697) in a solution of 0.1 M PB + 0.4% TX + 5% NGS for 2 h at room temperature with agitation. Positive control sections from previously verified PRV infected animals were included in each assay for assay reliability.

**Immunocytochemical controls**

Additional sections from each rat were processed in both the ER-ir + PRV-ir and the AR-ir + PRV-ir ICC assays, as above, but without any primary antibodies (zero primary antibody controls): this resulted in no labeling. Other sections were used as controls to test for cross-reactions between primary and secondary antibodies. The cross-reaction control sections were processed as above except that they were incubated with only one of the primary antibodies and both secondary antibodies, specifically: (i) RB132 and without 1D5, (ii) 1D5 and without RB132, (iii) the porcine anti-PRV antibody and without PG-21 and (iv) PG-21 and without the porcine anti-PRV antibody. These procedures, as expected, produced single-labeling only, indicating (i) that each primary antibody reacted normally with the appropriate secondary antibody and did not cross-react with the other secondary antibody, and (ii) that the secondary antibodies did not cross-react with each other. No dual-labeled or inappropriately single-labeled neurons were found in any of these control tissue sets.
**Imaging and Quantification**

For each brain section, digital serial photomicrographs were made at sufficiently high resolution to visualize individual labeled neurons (FIGURE 13). These photomicrographs were then melded together via a computer imaging program (Photoshop version 6.0) to make one image. The resulting photomicrograph was then magnified and section boundaries and major landmarks were traced onto it, as were the locations of cells staining positive for PRV-ir (with and without AR-ir or ER-ir). These tracings, with plotted cell locations, were mapped onto matching standard rat brain atlas plates by superimposing the tracings over the atlas plates via computer imaging and transferring the positions of the labeled cells to the plates; cells were then counted from these atlas plates using the regional boundaries and nomenclature of Swanson [146].

**RESULTS**

PRV successfully infected 11 of 20 rats, although there was considerable individual variation in the amount of infection per animal. In the diencephalic area of the brain, PRV labeled cells were located in several hypothalamic nuclei, notably the anterior hypothalamus (AHA), the bed nucleus of the stria terminalis (BST), the suprachiasmatic nucleus (SCN), the paraventricular nucleus hypothalamus (PVH), the median preoptic area (MEPO), the medial preoptic area (MPO), the medial preoptic nucleus (MPN), the retrochiasmatic hypothalamus, the lateral hypothalamus (LH), the dorsal medial hypothalamus (DM), the ventromedial hypothalamus (VMH) and the posterior hypothalamus (PH). Other areas exhibiting PRV infections were the premammallary
nucleus (PM), the arcuate nucleus (ARC), the ventral tegmental areas, the zona incerta (ZI), the periaqueductual gray (PAG) and the central nucleus of the amygdala (CEA) and the medial amygdala (MEA).

Because there was considerable individual variation in the extent of PRV-ir neuronal labeling among the eleven rats that developed infections, the four cases most heavily labeled by PRV-ir were mapped and quantified in entirety (TABLES 3 and 4). The remainder of the PRV-ir labeled cases (N = 7 rats) were not quantified because PRV-ir neuronal labeling was sparse (fewer than 5 PRV-ir neurons in the MPO).

**Distribution of PRV-ir and ER-ir Labeling**

Examples of ER-ir single-labeled neurons (FIGURE 14A), PRV-ir single-labeled neurons (FIGURE 14B) and PRV-ir + ER-ir dual-labeled neurons (FIGURE 14C) were found in all mapped cases. Injection of PRV into the prostate gland produced selective labeling of the brain, which is illustrated in two cases, the first of which (FIGURE 15) is more heavily labeled than the second (FIGURE 16); in both figures, PRV-ir single-labeled neurons (as shown in FIGURE 14B) are represented as open circles and PRV-ir + ER-ir dual-labeled neurons (as shown in FIGURE 14C) are represented as filled circles. PRV-ir labeling, with and without ER-ir, was consistently present in the MPO (including the MPN), the BST, PVH, ZI and in a continuum of cells in the perifornical area that extended through the LH to the PH. Sparser and/or inconsistent PRV-ir labeling also was noted in the CEA, MEA, VMH and PAG.
Distribution of PRV-ir and AR-ir Labeling

Examples of AR-ir single-labeled neurons (FIGURE 14D), PRV-ir single-labeled neurons (FIGURE 14E) and PRV-ir + AR-ir dual-labeled neurons (FIGURE 14F) were found in all mapped cases. The distribution of PRV-ir labeling, with or without AR-ir, matched very closely the distribution described above for PRV-ir, with or without ER-ir, even though different anti-PRV primary antibodies were used and is depicted for two cases (FIGURE 17, the more densely labeled case and FIGURE 18). PRV-ir single-labeled neurons are shown as open circles and PRV-ir + AR-ir dual-labeled neurons are shown as filled circles. To facilitate comparisons between the distribution of PRV-ir + AR-ir labeling and that of PRV-ir + ER-ir, FIGURES 15 and 17 are from one animal. FIGURES 16 and 18 are also from one animal (a different case from that plotted in FIGURES 15 and 17). PRV-ir labeling, with and without AR-ir, was consistently present in the MPO (including the MPN), BST, PVH, ZI and in a continuum of perifornical cells in the LH to the PH, and less consistently present and/or more sparse in the CEA, MEA, VMH and PAG.

Quantification of Dual Labeling

For each of the four mapped cases, TABLE 3 provides total counts of PRV-ir neurons and the percentages of those that also expressed ER-ir and TABLE 4 presents total counts of PRV-ir neurons and the percentages that were AR-ir. In the MPO, MPN, BST, and PVH there was a high degree of both ER-ir and AR-ir labeling in PRV-ir neurons.
DISCUSSION

After injection into the prostate gland, PRV, which is taken-up by both parasympathetic and sympathetic motor neurons [7], produced labeling in areas of the rat brain that are instrumental in autonomic function and several of these areas have previously been shown to mediate male mating behavior, specifically the MPO, BST, ZI and PAG. Moreover, in these areas and also in the PVH, the majority of cells staining positive for PRV-ir also expressed either ER-ir or AR-ir. This result supports the hypothesis and is consistent with the idea that, in the brain, a network composed of interconnected steroid-responsive neurons promotes the display of male copulatory behavior. The present findings reinforce earlier reports that mating-induced Fos-ir is colocalized with steroid receptors in neurons in these same brain areas [51].

The idea that mating behavior is controlled by a supraspinal neuronal network that integrates exogenous and endogenous cues is not new. Such a circuit has been described for the lordotic posture in females [41, 119] however; the hypothetical supraspinal circuit in males has received less attention. Convergent evidence from past research implicating brain areas that are important for the expression of male mating correlates with brain areas labeled by PRV-ir in this study, however, the pattern of information flow through this pathway or circuit and its steroid-responsiveness have yet to be fully described. In this study, by infecting the prostate gland with PRV, we labeled elements, principally in a forebrain diencephalic circuit, that presumably mediate both autonomic and motor aspects of male mating. Many of these PRV-ir neurons in the MPO, MPN, BST, ZI and PAG were found to be steroid responsive. The distribution of labeling from our study is
in agreement with previous studies that introduced PRV into the prostate gland [114, 178] or prostate gland and urinary bladder [107] but no other study has, to our knowledge, shown the steroid-sensitivity of the PRV-ir labeled neurons. This steroid responsiveness is an important feature of a supraspinal network that controls mating behavior. Thus, if such a circuit exists, it will show: (i) activity with mating, (ii) steroid sensitivity and (iii) connections with peripheral structures involved in mating. Our results are consistent with the identification of such a circuit.

It is universally accepted that the MPO plays a central role in the expression of vertebrate male sexual behavior. Lesions of the MPO eliminate male mating behavior [28, 56, 78] and stimulation of the MPO facilitates it [43, 44, 87, 153]. Not surprisingly, destruction of the MPN, a major subnucleus of the MPO also disrupts male mating [145]. Lesions of the BST and/or the stria terminals (st; a fiber tract that connects the amygdala with the MPO) interrupts male mating but to a lesser extent than do lesions of the MPO. Specifically, BST and st lesions generally affect the cadence of mating, resulting in increased frequencies of behaviors preceding ejaculation and longer latencies until ejaculation occurs [45, 48, 150, 177]. Lesions that include the ZI virtually eliminate male mating behavior [85]. Studies that combined PAG lesions with MPO stimulation suggest that the descending pathway for male sexual behavior passes through the PAG from the MPO [89].

PRV-ir labeling also occurred in some brain areas that traditionally have not been associated with male mating. For example, heavy PRV-ir occurred in the all cellular layers of the PVH and in a continuum of perifornical cells through the LH and PH. This
is not unexpected because these brain areas are involved in control of numerous autonomic targets and many sexual responses have an autonomic component, among them, vasodilatation, vasoconstriction, emission, secretions from seminal vesicles, contraction of the prostate, vas deferens and other reproductive organs. Interestingly, mating-induced Fos-ir has been reported in the PVH of male rats [8, 58, 70, 166]. Moreover, lesions of the parvocellular PVH are associated with a “decrease in seminal emission at the time of ejaculation” [2] and there are suggestions that the PVH might be involved in the supraspinal control of ejaculation [166]. Moreover, some PVH neurons project to the spinal nucleus of bulbocavernosus (SNB) [156, 157], a spinal cord area that regulates penile reflexes [16] and several studies have suggested that the descending oxytocinergic neurons of the PVH play a role in male sexual behavior [4, 23, 62].

The central tegmental field (also known as the posterior subparafascicular nucleus of the thalamus) (CTF/SPFp) and the MEA showed little or no PRV-ir. This was unexpected because both areas are known have direct reciprocal connections to the MPO, and both express mating-induced Fos-ir colocalized with steroid hormone receptors [51-53]. We expected to see dense PRV-ir in steroid-sensitive neurons of the MEA because previous studies from our laboratory demonstrated that estradiol (E2) acts in the MEA to promote sexual behavior, which is attenuated in the absence of E2 in the MEA. Specifically, blocking responses to E2 by inhibiting the conversion of E2 from T [60], or knocking-down the expression of ER-alpha receptors in the MEA [116] causes severe deficits in mating. Conversely, E2 implants to the MEA promote mating [59]. Moreover, it has been well documented that both pheromonal and chemosensory cues vital for
mating are processed in the amygdala [110]. These sensory related functions, which are hormone dependent, suggest that the amygdala interfaces with brain areas that mediate the motor aspects of mating and might modulate activity in the MPO. It is possible that, given more time, cells in the amygdala would have become PRV infected, but in this study they did not, perhaps because they are one or more synapses removed from regions (such as the MPO) that mediate motor aspects of mating.

A series of recent studies has defined a spinal ejaculation generating center in the lumbar spinal cord [149, 35], highlighting a set of neurons that are specifically activated with ejaculation. PRV studies have shown that these cells receive input from the prostate and bulbospongious muscle, supporting the idea that the spinal generating center coordinates emission and ejaculation [172, 173]. These lumbar neurons ascend the spinal cord and terminate in the CTF/SPFp, which has reciprocal connections to the MPO [52, 53]. The MPO is heavily reciprocally connected to the BST and MEA; thus, these ascending spinal neurons probably transmit ejaculatory information to the brain. Because CTF/SPFp neurons appear to be activated as a consequence of ejaculation, it is possible that such genital/somatosensory feedback information to motor brain regions controlling mounting behavior (such as the MPO) is indirect. In the present study, there might not have been sufficient time for cells in the CTF/SPFp to have become PRV infected if they are also one or more synapses removed from the MPO.

Mating behavior does not always end in ejaculation. Not all mounting behavior leads to intromission and not all mounts with intromission lead to ejaculation. Because gonadal steroid hormones must act in the brain for mounting to be initiated, presumably a
steroid-responsive brain network integrates exogenous sensory cues with the motor output needed to initiate mating behavior. This network, in all probability, synchronizes not only the autonomic motor neurons but also the skeletal motor neurons necessary for mating. Perhaps primary somatosensory feedback is constantly being received and integrated in the brain which, in turn, signals peripheral structures, coordinating mating postures and insemination. Such central-peripheral-central circuits and their mechanisms of hormonal regulation are not yet fully understood but Fos-ir labeling patterns and hormone manipulation studies have predicted a circuit for the initiation of mating made up of steroid-responsive neurons. The PRV-ir labeling patterns observed in this study provides evidence supporting this idea. Collectively, the results of this study suggest that we have begun to identify a steroid-responsive, supraspinal neuronal circuit that integrates and initiates the motor aspect of mating and that synchronizes autonomic responses with behavioral components.

ACKNOWLEDGEMENTS

This work partially fulfills the Doctor of Philosophy degree requirements of the Department of Biology, Georgia State University for G. G. Huddleston and was supported by National Science Foundation grant IBN 9876754 to the Center for Behavioral Neuroscience.
FIGURE 13
Method for preparation of maps describing the distribution of PRV-ir in the male rat brain. High resolution photomicrographs of sections were melded together via computer and the positions of labeled cells and major landmarks were drawn. These tracings were then superimposed onto standard rat atlas plates and the positions of PRV-ir cells were transferred to the atlas plates.
FIGURE 13
FIGURE 14
Examples of neuronal labeling:  A. ER-ir single-labeled neurons (blue arrows), B. PRV-ir single-labeled neuron (red arrow), C. PRV-ir + ER-ir dual-labeled neuron (green arrow), D. AR-ir single-labeled neuron, E. PRV-ir single-labeled neuron and F. PRV-ir + AER-ir dual-labeled neuron.
FIGURE 14
FIGURE 15

Distribution of PRV-ir + ER-ir in a heavily-labeled case. Open circles represent PRV-ir single-labeled neurons and closed circles represent PRV-ir + ER-ir dual-labeled neurons.
FIGURE 15, continued
FIGURE 16

Distribution of PRV-ir + ER-ir in a lightly-labeled case. Open circles represent PRV-ir single-labeled neurons and closed circles represent PRV-ir + ER-ir dual-labeled neurons.
FIGURE 16, continued
FIGURE 16, continued
FIGURE 17
Distribution of PRV-ir + AR-ir in a heavily-labeled case. Open circles represent PRV-ir single-labeled neurons and closed circles represent PRV-ir + AR-ir dual-labeled neurons.
FIGURE 17
FIGURE 17, continued
FIGURE 18

Distribution of PRV-ir + AR-ir in a lightly-labeled case. Open circles represent PRV-ir single-labeled neurons and closed circles represent PRV-ir + AR-ir dual-labeled neurons.
<table>
<thead>
<tr>
<th>CASE #:</th>
<th>9</th>
<th>24</th>
<th>11</th>
<th>26</th>
<th>SUMMARY (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cell #</td>
<td>Total cell #</td>
<td>Total cell #</td>
<td>Total cell #</td>
<td>Total cell #</td>
</tr>
<tr>
<td>BRAIN AREA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO @ -0.46</td>
<td>N/A</td>
<td>31</td>
<td>74%</td>
<td>15</td>
<td>87%</td>
</tr>
<tr>
<td>MPO @ -0.51</td>
<td>29</td>
<td>79%</td>
<td>60</td>
<td>77%</td>
<td>11</td>
</tr>
<tr>
<td>MPN @ -0.46</td>
<td>N/A</td>
<td>8</td>
<td>75%</td>
<td>4</td>
<td>100%</td>
</tr>
<tr>
<td>MPN @ -0.51</td>
<td>13</td>
<td>69%</td>
<td>12</td>
<td>92%</td>
<td>1</td>
</tr>
<tr>
<td>BST @ -0.46</td>
<td>N/A</td>
<td>12</td>
<td>67%</td>
<td>6</td>
<td>50%</td>
</tr>
<tr>
<td>BST @ -0.51</td>
<td>55</td>
<td>69%</td>
<td>20</td>
<td>55%</td>
<td>4</td>
</tr>
<tr>
<td>BST @ -1.08</td>
<td>3</td>
<td>33%</td>
<td>0</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td>PVH @ -1.08</td>
<td>10</td>
<td>40%</td>
<td>4</td>
<td>100%</td>
<td>N/A</td>
</tr>
<tr>
<td>PVH @ -1.78</td>
<td>2</td>
<td>50%</td>
<td>10</td>
<td>60%</td>
<td>8</td>
</tr>
<tr>
<td>CEA @ -1.78</td>
<td>3</td>
<td>67%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>CEA @ -2.45</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td>MEA @ -2.45</td>
<td>1</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td>ZI @ -1.78</td>
<td>1</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>ZI @ -2.45</td>
<td>17</td>
<td>82%</td>
<td>12</td>
<td>17%</td>
<td>N/A</td>
</tr>
<tr>
<td>VMH @ -2.45</td>
<td>6</td>
<td>100%</td>
<td>3</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td>PAG @ -4.45</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>PAG @ -4.60</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PAG @ -5.65</td>
<td>10</td>
<td>70%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CASE #:</td>
<td>9</td>
<td>24</td>
<td>11</td>
<td>26</td>
<td>SUMMARY (Mean ± SEM)</td>
</tr>
<tr>
<td>--------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>Total cell #</td>
<td>% AR-ir</td>
<td>Total cell #</td>
<td>% AR-ir</td>
<td>Total cell #</td>
</tr>
<tr>
<td>BRAIN AREA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO @ -0.46</td>
<td>19</td>
<td>58%</td>
<td>N/A</td>
<td>14</td>
<td>36%</td>
</tr>
<tr>
<td>MPO @ -0.51</td>
<td>22</td>
<td>36%</td>
<td>25</td>
<td>52%</td>
<td>5</td>
</tr>
<tr>
<td>MPN @ -0.46</td>
<td>11</td>
<td>73%</td>
<td>N/A</td>
<td>9</td>
<td>33%</td>
</tr>
<tr>
<td>MPN @ -0.51</td>
<td>7</td>
<td>71%</td>
<td>5</td>
<td>20%</td>
<td>0</td>
</tr>
<tr>
<td>BST @ -0.46</td>
<td>29</td>
<td>48%</td>
<td>N/A</td>
<td>13</td>
<td>46%</td>
</tr>
<tr>
<td>BST @ -0.51</td>
<td>46</td>
<td>65%</td>
<td>19</td>
<td>84%</td>
<td>13</td>
</tr>
<tr>
<td>BST @ -1.08</td>
<td>N/A</td>
<td>0</td>
<td>0%</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>PVH @ -1.08</td>
<td>N/A</td>
<td>3</td>
<td>67%</td>
<td>N/A</td>
<td>6</td>
</tr>
<tr>
<td>PVH @ -1.78</td>
<td>38</td>
<td>37%</td>
<td>18</td>
<td>33%</td>
<td>N/A</td>
</tr>
<tr>
<td>CEA @ -1.78</td>
<td>18</td>
<td>44%</td>
<td>2</td>
<td>50%</td>
<td>N/A</td>
</tr>
<tr>
<td>CEA @ -2.45</td>
<td>0</td>
<td>0%</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>MEA @ -2.45</td>
<td>8</td>
<td>63%</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>ZI @ -1.78</td>
<td>1</td>
<td>100%</td>
<td>2</td>
<td>50%</td>
<td>N/A</td>
</tr>
<tr>
<td>ZI @ -2.45</td>
<td>27</td>
<td>74%</td>
<td>N/A</td>
<td>N/A</td>
<td>4</td>
</tr>
<tr>
<td>VMH @ -2.45</td>
<td>4</td>
<td>75%</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>PAG @ -4.45</td>
<td>17</td>
<td>82%</td>
<td>0</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td>PAG @ -4.60</td>
<td>N/A</td>
<td>3</td>
<td>67%</td>
<td>N/A</td>
<td>6</td>
</tr>
<tr>
<td>PAG @ -5.65</td>
<td>13</td>
<td>69%</td>
<td>3</td>
<td>33%</td>
<td>0</td>
</tr>
</tbody>
</table>
CHAPTER 5

GENERAL DISCUSSION

The findings presented here suggest that E2 plays an important role in the expression of male rat sexual behavior. These studies extend previous reports that: (1) local aromatization of T to E2 in the brain is necessary for mating behavior to occur [44, 83, 108, 140, 141, 158], (2) mating-induced Fos-ir is colocalized with ER and AR in areas previously shown to exhibit steroid sensitivity [8, 36, 51, 70] and (3) it is likely that a steroid-responsive, supraspinal network coordinates male copulation. Furthermore, our studies partially support earlier reports that implicate ER-α as the behaviorally relevant form of ER [112, 129]. We found that knockdown of ER-α in the MPO attenuates mating behavior whereas, disabling ER-α in the MEA has no effect. This finding suggests differential mechanisms of ER action in the brain. Moreover, to our knowledge, we report for the first time that stimulation of a form of mER is sufficient to elicit mating behavior, at least in the MPO but interestingly, not in the MEA, and to our knowledge, this is also the first report that the E2 remains conjugated to BSA in vivo. Collectively, there are four main conclusions from these studies: (1) E2 acts in the brain to mediate expression of male copulatory behavior and plays a larger role than was previously appreciated, (2) the mechanisms by which E2 acts to promote mating varies in different brain areas, (3) depending on the brain area, a cell-surface stimulation of ER is sufficient for mating behavior to occur, and (4) supportive evidence was obtained for the existence
of a steroid-responsive neural network that governs male sexual behavior, one that is similar to that described in females that mediates lordosis [41, 119].

In chapter 1 we hypothesized that the knockdown of ER-α in either the MPO or the MEA would suppress mating. This hypothesis was partially supported: ER-α appears to mediate responses to E2 in the MPO, but ER-α is not necessary in the MEA for sexual behavior to occur. In 1996, a novel form of ER was discovered; ER-β [76]. Because ER are reported to dimerize upon ligand binding [147], there is the possibility of either ER-α or ER-β homodimers or heterodimers. There are few if any studies of the function of hetero- vs. homodimers. Nevertheless, one may speculate that in the MPO there must be either homodimerization of ER-α, or heterodimerization (or both) for mating to occur, providing the mechanism by which behavior is expressed is via dimerization and selective transcription. If the behavioral mechanism of ER is via a membrane receptor, then dimerization of receptors might be crucial in some brain areas, but not others. Thus, we need to begin to ask new and different questions concerning the role of hormones and receptors in the display of mating behaviors.

Historically, E2 has been described as a transcription factor that, upon binding its cytoplasmic receptor, translocates to the nucleus and acts as a transcription factor [64, 68, 93, 164]. Although it has been known for several years that E2 exerts rapid physiological effects (within seconds) on MPO [65] and MEA [106] neurons in vitro, only recently have reports of rapid effects of E2 become mainstream. Initially, these rapid effects of E2 (assumed to be non-genomic effects), were described as causing virtually instantaneous cell-signaling cascades. We now know that the rapid, cell-signaling effects of E2 and
genomic actions of E2 are not necessarily mutually exclusive. There is evidence to show that the rapid/cell signaling effects of E2 might, in fact, culminate in gene transcription [98, 160]. In studies aimed at finding and characterizing mERs there are some reports suggesting that mER is either ER-α or an isoform of ER-α [88, 101, 159]. Whatever the form of mER, we were interested in the possibility that mER might mediate sexual behavioral responses. To that end, in Chapters 2 and 3 we used BSA-E2 to stimulate the mERs that might be associated with neurons of the MPO and MEA. BSA is a large protein that will not permeate the plasma membrane and as shown in chapter 2, there is no cleavage of the bond conjugating BSA to E2 \textit{in vivo}. Because the knockdown of ER-α in the MEA did not interfere with mating behavior, we hypothesized that the recently described, novel mER, GRP30 [128, 148] or some similar receptor, might mediate the copulatory behavior in the MEA. Conversely, even though there are reports that mERs might be ER-α, we hypothesized that stimulation of mER in the MPO would not be sufficient to elicit mating behavior. Neither hypothesis was supported. Instead stimulation of mER (via BSA-E2) in the MPO supported male mating behavior at significantly higher levels than controls on all measured indices (Figure 6). However, membrane-stimulation by BSA-E2 of the MEA was not sufficient to maintain mating behavior. Our conclusions from these two studies are: (1) that E2 works through different mechanisms in the MEA and MPO, (2) that a cell surface action of E2 in the MPO is sufficient for mating behavior to occur, but (3) an intracellular, non-ER-α mechanisms is indicated in the MEA. In addition, in Chapter 3, we performed the converse of the experiment presented in the Appendix. By chronically administering Fadrozole
bilaterally to the MEA of intact male rats, we showed that mating behavior is attenuated (Figure 9), supporting and extending the evidence that E2 acts in the MEA to promote male sexual behavior (see Appendix).

Because gonadal steroid hormones act on brain hypothalamic and limbic sites to promote male mating behavior, and because mating-induced Fos-ir is colocalized with ER-ir and AR-ir in these sites [51], it is reasonable to hypothesize that these brain areas act together, under the influence of hormones, to mediate the display of copulation. The lordosis response in females and the ejaculatory component of male sexual behavior are reflexes that are under the control of the spinal cord. Supraspinal modulation and control of the lordotic reflex has been described [41, 119]. Presumably there is a similar network in males that modulates the ejaculatory reflex. If such a neural network exists, it must, by definition, have the following characteristics; (1) steroid responsiveness, (2) connectivity with peripheral structures that are involved in sexual behavior, (3) terminals in brain areas reported to be involved in sexual behavior (4) an ability to synchronize and coordinate the physiological and behavioral responses associated with copulation.

We reasoned that one could describe such a circuit by microinjecting PRV into the prostate gland, a gland whose only known function is fluid secretion and expulsion during mating. PRV is a transynaptic retrograde tract tracer taken up by autonomic motor neuron terminals [144, 7]. Inside the infected neuron, self-replication occurs and the virus crosses synapses in a retrograde, chain-like fashion [19, 20]. Furthermore, by using dual labeling ICC with an anti-PRV antibody and either an anti-AR antibody or an anti-ER antibody, we hypothesized that we could visualize an entire multisynaptic,
steroid-responsive neural network that coordinates the expulsion of prostatic fluid and which might mediate with the behavioral patterns associated with mating.

PRV-ir + AR-ir and PRV-ir + ER-ir labeled cells showed a common distribution. This extends and supports the idea that both androgens and estrogens mediate mating. Thus, neither DHT nor E₂ alone is as effective in maintaining or reinstating mating behavior as is either T alone or a combination DHT and E₂ [63, 97]. Dual labeling was most consistently present in the MPO (including at least one of its subdivisions), the MPN, the BST, PVH, and ZI and in a continuum of cells surrounding the fornix that extended through the LH to the PH (Figures 15-18 and Tables 3-4). With the possible exception of the LH and PH, each of these areas has been shown to be important in the display of male copulatory behavior [63, 97]. Unexpectedly, labeling in the CTF/SPFp and the MEA was light and inconsistent if present at all. The CTF/SPFp and MEA contains steroid sensitive neurons [51, 52] and both are part of an ascending neuronal pathway that is believed to transmit ejaculatory information from the spinal cord to the brain [35]. Both areas are reciprocally connected to the MPO, thus we cannot explain their lack of PRV-ir labeling. Future studies will be necessary to determine both additional elements in the circuit described here and to test that it mediates male mating behavior as proposed. Nevertheless, this study supports the idea of a central-peripheral-central hormonally regulated circuit. In conclusion, we believe that we have begun to identify a circuit that integrates and initiates the motor aspect of mating and that synchronizes autonomic responses with behavioral responses associated with mating.
The overall conclusions from these studies are that: (1) \(E_2\) acts in the brain to mediate expression of male copulatory behavior and plays a larger role than has been previously appreciated, (2) the mechanisms by which \(E_2\) acts to promote mating varies in different brain areas, (3) depending on the brain area, a cell-surface stimulation of ER is sufficient for mating behavior to occur, and (4) supportive evidence was obtained for the existence of a steroid-responsive neural network that governs male sexual behavior, one that is similar to that described in females that mediates lordosis [41, 119].

The significance of these studies is that they begin to elucidate how \(E_2\) acts in the brain to mediate behavioral responses. This extends considerably our understanding of how hormones affect mating because previous research revealed WHERE, but not HOW, hormones act on the brain. Understanding HOW hormones act is important because of the many roles that steroids play in the regulation of health and in the environment.
LIST OF REFERENCES


81. Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH: Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha. Proc Natl Acad Sci USA 2004; 101: 17126-17131.


96. McGinnis MY, William GW, Lumia AR: Inhibition of male sexual behavior by androgen receptor blockade in preoptic area or hypothalamus, but not amygdala or septum. Physiol Behav 1996; 60: 783-789.


APPENDIX

ESTRADIOL IN THE MALE RAT AMYGDALA FACILITATES MOUNTING BUT NOT EJACULATION

Gloria G. Huddleston, Richard P. Michael, Doris Zumpe & Andrew N. Clancy,

ABSTRACT

HUDDLESTON, G.G., R.P. MICHAEL, D. ZUMPE AND A. N. CLANCY, Estradiol in the male rat amygdala facilitates mounting but not ejaculation. PHYSIOL BEHAV 79 239-246, 2003. Mating activates estrogen sensitive neurons in several regions of male rat brain, including the medial amygdala (MEA). Infusion of the aromatase inhibitor, Fadrozole, into the MEA reduced mating, presumably by inhibiting conversion of testosterone (T) to estradiol (E2). We investigated whether administering E2 locally into the amygdala (AMG) would maintain sexual behavior in male rats given systemic Fadrozole to eliminate E2 elsewhere in the brain. Gonadally intact male rats were divided into two matched groups, based on ejaculatory performance in weekly tests with receptive females. All males received 0.29 mg/kg/day Fadrozole s.c. and bilateral implants to AMG. E2-in-AMG males (N = 6 experimentals) received implants tipped with a cured mixture of E2 in Silastic Medical Adhesive, whereas Vehicle-in-AMG males (N = 6 controls) received implants tipped with cured adhesive alone (without E2). In E2-in-AMG males, postoperative mount and intromission frequency did not differ significantly from pretreatment baseline levels, but ejaculation frequency declined significantly (p<0.01). Conversely, in Vehicle-in-AMG males, postoperative mounts and intromissions (p<0.01) and ejaculations (p<0.01) declined significantly. Postoperative mount and intromission frequency of Vehicle-in-AMG males was significantly lower than that of E2-in-AMG males (p<0.01), but ejaculation frequency did not differ significantly between groups. This suggests that E2-sensitive AMG neurons are important for sexual arousal but not ejaculatory performance.
INTRODUCTION

The display of copulatory behavior by male mammals is dependent on testicular hormones acting on the brain as well as on peripheral structures. Lesions, both electrical and chemical, have defined a number of brain sites that are believed to be important for sexual behavior. Because neurons in many of these same sites contain androgen receptors (AR), estrogen receptors (ER) or AR colocalized with ER [1,2] several studies have investigated the effects of hormone deprivation, administration, and receptor blockade on behavior. Testosterone (T) and its metabolites, dihydrotestosterone (DHT) and estradiol (E₂), have been shown to affect the brain through receptor mechanisms without which, mating does not occur [3]. It is generally agreed that both T and E₂ are necessary for full expression of male copulatory behavior, although neither the anatomical framework nor the hormonal requirement(s) for male sexual behavior is fully understood [97].

Immunocytochemical (ICC) studies have shown that Fos, the protein product of the early immediate gene c-fos, is an accepted correlational marker for neuronal activation [105]. The same areas identified by lesion and stimulation methods as being important for sexual behavior show increased levels of Fos-immunoreactivity (ir) associated with mating [8]. In male hamster [70] and male rat [36, 51], the medial preoptic area (MPO), bed nucleus of the stria terminalis (BST), medial amygdala (MEA) and central tegmental field all contain neurons in which mating-induced Fos-ir is colocalized with either AR-ir or ER-ir alone, or with both AR-ir and ER-ir.
In a study measuring male rat mating and the neural formation of E₂ from T, 0.25 mg/kg/day s.c. of the aromatase inhibitor Fadrozole (CGS-15949A, CIBA-Geigy Corporation, Summit, NJ) significantly lowered copulatory behavior and inhibited by 95% the manufacture of ³H-E₂ from ³H-T in several areas of the brain [12]. A follow-up study, using an ICC assay based on the observation that E₂ suppresses H222 ER-ir labeling in the brain [6, 29], confirmed that 0.25 mg/kg/day s.c. Fadrozole blocked synthesis of E₂ from T [30]. Consequently, castrated male rats given Fadrozole and T, but not those maintained on T alone, exhibited abundant H222 ER-ir labeling throughout the brain [30].

The MPO, one of the areas that colocalizes AR-ir and ER-ir, has been regarded as an integration site, essential for the display of male sexual behavior [97]. Gonadally intact male rats with bilateral E₂ implants to the MPO plus 0.25 mg/kg/day s.c. Fadrozole expressed all components of sexual behavior at significantly higher levels than did controls, whose mating was significantly impaired, although the behavior of the E₂-implanted males was not robust [33]. Furthermore, H222 ER-ir labeling confirmed that E₂ had been confined exclusively to the MPO. Virtually no H222 ER-ir labeling occurred in the MPO of experimental males, although it was abundant in the MPO of controls and elsewhere in the brains of both groups [33].

AR-ir and ER-ir are also colocalized in the MEA [51], an area that is reciprocally connected to the MPO in the rat [18] and in the hamster [39]. Estradiol implants in the corticomedial amygdala of castrated male rats restored mounting behavior, however, intromissions and ejaculations were not measured [126]. Similarly, in hamsters, E₂
implanted into the posterior MEA of castrate males increased mounts and non-copulatory sexual behaviors [167]. We hypothesized that confining E2 to the MEA of male rats with a natural testosterone source would maintain all components of male sexual behavior at robust levels. Therefore, gonadally intact male rats were treated with 0.29 mg/kg/day s.c. Fadrozole (a higher dose than used in previous studies) and divided into two groups: experimentals (E2-in-AMG) received bilateral implants of E2 directed at the MEA, and controls (Vehicle-in-AMG) received bilateral implants of vehicle directed at the MEA. H222 ER-ir labeling assessed the spread of E2 in the brain.

EXPERIMENTAL PROCEDURES

Animals. Male (465-644 g) and female (276-300 g) Sprague-Dawley rats, at least 90 days of age, were obtained from Charles River Laboratories or were bred in the laboratory. Males and females were housed separately in plastic cages with free access to food and water in a climate-controlled rat colony under a 14:10 hour reverse light:dark cycle (lights off at 1000 hours). 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).

Ten female rats were injected i.p. with 62.4 mg/kg sodium pentobarbital and then ovariectomized through a single midline abdominal incision. At the same time, each female was also implanted s.c. with a 10 mm Silastic capsule filled with crystalline E2. Penicillin (0.1ml i.m. benzathine penicillin G + procaine penicillin G) and Banamine (0.3 ml i.m.) was given post-operatively to relieve surgical discomfort and to reduce the
likelihood of infection. Females were allowed a minimum of seven days recovery time before the start of behavioral testing. Stimulus (E2-maintained) females received 1.0 mg progesterone s.c. (1mg progesterone/0.2 ml sesame oil) 4-6 hours before being tested with males.

Male rats were screened for sexual behavior in five weekly 30 minute mating tests to determine their suitability for inclusion in the experiment. Thereafter, twelve proven ejaculators were matched based on ejaculatory performance and assigned to two groups: E2-in-AMG (N = 6) or Vehicle-in-AMG (N = 6).

**Sexual behavior tests.** During sexual screening and postoperative mating tests, each male was paired with a receptive female for 30 minutes in a 22 x 44 x 50 cm testing arena under dim red light. Tests began about 4 hours after lights off. A laptop computer was used to tally mount frequency (MF), intromission frequency (IF) and ejaculation frequency (EF). We also measured mount latency (ML - time from the start of the test until the first mount or intromission or 1800 seconds if no mounts occurred), ejaculation latency (EL - time from the first mount or intromission until the first ejaculation or 1800 seconds if no ejaculations occurred) and the post-ejaculatory interval (PEI – time between an ejaculation and the next mount or intromission or 1800 seconds if no ejaculations occurred).

**Estradiol and Fadrozole administration to males.** Swanson’s atlas [146] was used to provide coordinates of the MEA: horizontal skull coordinates were –2.8 mm ap, 3.9 mm
ml, and –9.3 mm dv. At the time of surgery, each male was anesthetized with sodium pentobarbital, 62.4 mg/kg i.p., and given atropine sulfate, 1.35 mg/kg i.m. Bilateral 25 gauge stainless steel implants were stereotaxically placed into the MEA and secured with jewelers screws to act as anchors for cranioplastic cement. Antibiotic powder was applied and clips were used to close the incision. E$_2$-in-AMG males received air dried, ethylene oxide sterilized MEA implants whose tips had been coated with Silastic Medical Adhesive (Dow Corning) mixed with E$_2$ (50.1 mg β-estradiol [Sigma] and 457 mg adhesive). Vehicle-in-AMG males received implants whose tips had been coated with Silastic Medical Adhesive only (no E$_2$). In addition, all males were given Fadrozole s.c. via ALZET osmotic minipumps (model 2004, 200 μl capacity). Pumps, loaded with 27.18 mg Fadrozole/ml saline, dispensed the solution at the rate of 6 μl/day for 28 days; therefore, rats received a Fadrozole dose of 0.29 mg/kg/day. Minipumps were filled, weighed, and immersed in physiological saline at 37°C for 48 hours prior to surgery, this ensured full pumping activity at the time of implantation. Animals were given 5 to 7 days recovery time before weekly behavioral testing was resumed. Twenty-six days after implantation, each minipump was replaced under sodium pentobarbital anesthesia with a second pump identical to the first, so that behavioral testing could be extended. The testing schedule ensured a minimum of 5 days of recovery after minipump replacement before the next behavioral test. Fluid levels in the pumps were checked at the end of the study to determine that there had been no pump failures; none occurred.
**Immunocytochemical assays.** When the testing period was concluded, males were killed with an overdose of sodium pentobarbitol (150 mg/kg i.p.) and perfused transcardially for 5 minutes with isotonic saline followed by a minimum of 300 ml 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 over night in perfusion fixative. Brains were removed from skulls the next day and stored in 30% sucrose in 0.1M PB for approximately 48 hours.

Coronal frozen brain sections (40 μm) were collected into 0.1 M PB. Free floating sections through the diencephalon were obtained from each brain and matched sets of sections from both groups of males were processed concurrently using the same aliquots of diluted antisera and immunoreagents to minimize interassay variations. Sections were incubated for 24 hours in a blocking solution containing 3% normal goat serum and 0.2% Triton X-100 in 0.1M PB. Sections were then incubated for 48 hours in 1 μg/ml H222 (Abbott Laboratories), a rat monoclonal anti-ER antibody raised against the steroid-binding domain of a human ER protein fragment [55]. H222 anti-ER labeling is reported to be inhibited in brain by the presence of E2; conversely, labeling is present in the absence of E2 [6, 29, 30, 55]. The tissue was given three 5 minute washes in 0.1M PB and then incubated for 2 hours in a goat anti-rat gold-conjugated secondary antibody (BBI international, 1/250 dilution), followed by three 5 minute washes in deionized water, and a 20 minute incubation in silver enhancement reagents (BBI International) following the manufacturer’s directions. After a final PB wash, sections were mounted on clean gel-albumin coated slides, cleared of salts and coverslipped using DPX.
There are problems with the use of the H222 anti-ER antibody in rat tissues [6] due to the fact the H222 antibody was raised in rat [55]. The gold-conjugated secondary antibody used in this study was selected because it was the least cross-reactive in rat brain of several secondary antibodies we screened.

Quantification and statistics. H222-ir immunolabeling was mapped and quantified using Bioquant software (R & M Biometrics). The numbers of H222 ER-ir neurons were counted in microscopic fields measuring 180 X 130 μm in sections from the MEA, MPO and ventromedial hypothalamus (VMH). Histological data were analyzed by Mann Whitney U tests [133].

Because a male that intromits necessarily mounts, but a male that mounts does not necessarily intromit, to find all sexually active animals we summed MF and IF for each rat and performed statistics on combined mounts and intromissions, as well as on EF (Fig 19). Behavioral data from all pretreatment and postoperative tests were assessed with repeated measures (group X trial) analysis of variance and Tukey HSD post hoc tests [69]. Two-tailed probability levels are reported in all cases.

RESULTS

Male Sexual Behavior

Table 5 gives mean ± SEM MF, IF, ML, EL and PEI, and counts of the numbers of rats that were sexually active by mounts, intromissions or ejaculations.
Mounts and intromissions. Both groups showed high levels of mounting and intromission during baseline before implantation, and there were no significant differences between them. After implantation, mounts and intromissions declined significantly in males with vehicle in the amygdala compared to those with E₂ in the amygdala $F(8, 80) = 5.23$, group by trial interaction, ($p<0.001$) (Fig. 19 top). During the first post-implantation treatment test, each group showed a significant decrease in these behaviors compared to baseline ($p<0.01$). Thereafter, mounting and intromission recovered in males with E₂ implants, and in several post-implantation treatment trials were not significantly different from baseline levels. In contrast, mounting and intromission by males with vehicle in the amygdala remained significantly lower than baseline levels in all post-implantation treatment trials ($p<0.01$), and in several post-implantation treatment trials were significantly lower than those of males with E₂ in the amygdala ($p<0.01$) (Fig. 19 top).

Ejaculations. Both groups exhibited high and statistically indistinguishable levels of ejaculation in pretreatment baseline tests (Fig. 19 bottom). During the post-implantation treatment period, EF declined significantly in both groups $F(8,80)= 3.40$, trial main effect, ($p<0.002$), and did not recover. In most post-implantation trials there were no consistent differences between E₂-implanted and vehicle-implanted males.
Histology

Implant placements. Nissl sections showed that implant tips were widely distributed throughout the amygdala (AMG). Locations of implants are depicted on standard atlas plates in Fig. 20.

H222 ER-ir labeling. Because E₂ interferes with H222 ER-ir labeling in the brain [6, 29, 30, 55], it was used to verify (i) that s.c. Fadrozole treatment inhibited formation of E₂ from T in the brain, and (ii) that E₂ was confined to the AMG of experimental males. There was abundant H222 ER-ir labeling in the MPO (Fig. 21 A and B) and VMH (not shown) of both groups, as well as throughout the AMG of vehicle-implanted males (Fig. 21 D), suggesting that Fadrozole s.c. blocked the local conversion of T to E₂ in the brain. In the MEA (ap = -2.85, ml = 3.50, dv = 8.50, Swanson atlas [146]), H222 ER-ir labeling was substantially reduced or eliminated in E₂-implanted males (Fig. 21 C); there were significantly fewer labeled neurons in these males than in vehicle-implanted males, 12.50 ± 7.16 versus 162.67 ± 58.73, respectively (U =3, n₁ = 6, n₂ = 6, p<0.02). In contrast, there were no significant differences between the E₂-in-AMG and the Vehicle-in-AMG males in the numbers of H222 ER-ir neurons in either the MPO, (267.67 ± 27.71 versus 209.00 ± 49.84, respectively) or the VMH (27.50 ± 3.69 versus 35.17 ± 3.23, respectively). The scarcity of labeling in the MEA and elsewhere in the AMG of E₂-in-AMG males was presumably due to the presence of E₂ from the implants, and the occurrence of labeling in the MPO and VMH of these males suggests that the exogenous E₂ had not diffused from the AMG to the MPO or VMH.
DISCUSSION

Gonadally intact, sexually experienced male rats receiving Fadrozole s.c. together with E2 implants bilaterally to the AMG mounted and intromitted in most of the post-implantation tests, and their behavior was not significantly different from pretreatment baseline levels. Conversely, males that were treated with Fadrozole s.c. and received vehicle implants bilaterally to the AMG virtually ceased mating (Fig. 19). Ejaculatory performance was not maintained by either treatment.

In male mammals, T is enzymatically converted both centrally and peripherally by 5-alpha reductase to DHT and by aromatase to E2 [163]. Male copulatory behavior is restored or maintained in castrates by exogenous T [174] or by combined treatment with DHT and E2 [9, 84, 140, 158]), but not with E2 alone or with DHT alone [84, 92, 151, 163]. Moreover, brain areas shown to be important for the display of male sexual behavior as evidenced by the expression of Fos-ir (a marker for neuronal activity [131]), including the MEA, have also been shown to contain populations of neurons that are sensitive to androgens as well as other neurons that are sensitive to estrogens [41, 50, 52, 54] or both [51].

Previous work has shown the importance of E2-sensitive neurons of the MPO, an area believed to be necessary for the display of male sexual behavior. Bilateral irrigation of the MPO with Fadrozole significantly decreased mounts, intromissions and ejaculations in gonadally intact male rats, apparently because the MPO had been deprived of E2 derived from T [32]. Conversely, in a second study in which Fadrozole was administered s.c. to gonadally intact males and E2 was implanted bilaterally into the
MPO, levels of mounting and intromission and, to a lesser extent ejaculation, were maintained, suggesting that E₂ sensitive neurons in the MPO contribute to all components of mating [33].

Bilateral lesions of the MEA cause severe deficits in male rat mating behavior [71], suggesting that the MEA is also an area of critical importance in the display of male copulatory behavior. In the present study, the same protocol used to study the role of the MPO, namely, systemic Fadrozole combined with intracerebral E₂ implantation, was used here to study the AMG. Because a pilot study [31] showed that mating was significantly inhibited in gonadally intact males receiving bilateral Fadrozole in the MEA, we hypothesized that local administration of E₂ to the MEA of gonadally intact males with normal circulating testosterone levels would maintain mounting, intromission and ejaculation. Testicular T was present in the rats but its aromatization to E₂ was blocked in the MPO, VMH, MEA and presumably elsewhere in the brain by the administration of systemic Fadrozole, which significantly suppressed mating. The effect of Fadrozole was partially reversed by implanting the brain with exogenous E₂ aimed at the MEA, which maintained mounts and intromissions at pretreatment levels, but ejaculation was not maintained. Follow-up brain histology suggested, however, that E₂ was probably present throughout the AMG.

There was substantial variation in the locations of implants in the AMG. H222 ER-ir labeling in brain is inhibited by E₂ [6, 29] and E₂-in-AMG males had significantly less H222 ER-ir labeling of the MEA than Vehicle-in-AMG males. Moreover, little H222 ER-ir labeling was observed elsewhere in the AMG of E₂-in-AMG males, but was
abundant throughout the AMG of controls. Thus, it is likely that much of the AMG was exposed to E2 in the E2-in-AMG group whereas little, if any, E2 was present in the AMG of Vehicle-in-AMG controls. Estradiol apparently did not diffuse to brain sites remote to the AMG, such as the MPO and VMH. We infer this because H222 ER-ir was abundant in the MPO and VMH of both groups. There were no significant differences between groups in the numbers of H222 ER-ir MPO or VMH neurons, suggesting that Fadrozole inhibited the aromatization of T to E2 throughout the brain. Accordingly, we cannot draw conclusions about where E2 acts in the AMG to facilitate mounting and intromission, although the histological data are consistent with the interpretation that E2 sensitive AMG neurons are important for sexual arousal but not for ejaculatory performance.

Between test weeks 4 and 5, minipumps were changed to extend s.c. Fadrozole treatment. Behavioral performance on test week 5 may have been influenced by minipumps that had not fully stabilized. The data from week 5 are atypical yet interesting because they tend to rule out the possibility that incidental brain damage caused by the implantation procedures compromised sexual behavior. This is because most of the Vehicle-in-AMG controls mated to ejaculation on test week 5, which contrasts with an otherwise statistically significant, progressive decline in mating (Fig. 19 and Table 5). Because incidental brain damage was similar in the E2-in-AMG and Vehicle-in-AMG groups, it cannot account for behavioral differences between experimentals and controls. Consequently, these findings, in males with functional testes not compromised due to lack of circulating androgens, suggest that estrogen, acting in the amygdala, is necessary for sexual arousal (as reflected by mounting and intromission),
but not for ejaculation. These findings also suggest that estrogen is important for ejaculatory performance, perhaps by acting in spinal and/or brain areas outside the amygdala.

These findings extend previous reports showing that intracranial E2 administration to the AMG partially restored mounting in castrated male rats [126] and hamsters [167], but not to pre-castration levels. Testosterone may also be needed for normal levels of sexual behavior, since other studies showed that T implants to the male hamster MEA restored mounts and intromissions or mounts alone [168, 169]. The findings from the present study suggest that such effects may depend on the aromatization of T to E2 in the AMG, because Vehicle-in-AMG males (exposed to testicular T but not to E2) virtually ceased mating, whereas E2-in-AMG males that had circulating T together with E2 in the AMG mounted and intromitted at pretreatment levels.

This study raises questions concerning the cell types and brain areas that mediate specific components of mating behavior. One might interpret these results to imply that the AMG is a site that controls mounting and intromission, and that the expression of these behaviors is E2 dependent. If this is the case, then ER-containing cells in the AMG are not unique because other brain areas also contain E2-responsive neurons that contribute to mounting and intromission, e.g. the MPO [32, 33]. Redundancy might explain why disabling different individual brain areas results in the same behavioral display [40], although often the quality of the behavior varies. Thus, redundancy does not necessarily imply equal facilitation of sexual behavior among different brain nuclei [171]. This study, together with previous findings [31-33], suggests that E2 acts in the
AMG and MPO to mediate mating, which suggests that a network of E$_2$-sensitive neurons are important for male sexual behavior. Questions remain as to whether the AMG and MPO function as separate entities or as a unit [40, 74, 171] to mediate male sexual behavior. Findings from this study suggest that E$_2$-sensitive AMG neurons mediate sexual arousal or motivation but not ejaculatory performance.

ACKNOWLEDGEMENT

This work partially fulfills the Masters of Science degree requirements of the Department of Biology, Georgia State University for G. G. Huddleston and was supported by USPHS grant MH 19506, Whitehall Foundation grant J96-24 and NSF grant IBN 9876754, administered by the Center For Behavioral Neuroscience. We gratefully acknowledge the expert technical assistance from Frederick Cawthon and Marcelia Maddox.
FIGURE 19

Effects on the numbers (mean ± SEM) of mounts and intromissions (top) and ejaculations (bottom) of implanting E₂ (closed circles and solid lines) or vehicle (open circles and interrupted lines) bilaterally into the AMG of intact male rats receiving Fadrozole s.c. In E₂-implanted males, post-implantation levels of mounts and intromissions remained high, but ejaculations decreased significantly ($p<0.01$) in most post-implantation tests. In vehicle-implanted males, there was a significant ($p<0.01$) post-implantation decline in mounts and intromissions as well as in ejaculations.
FIGURE 19

Mounts & Intromissions

- E₂-in-AMG
- Vehicle-in-AMG

* between group difference (p<0.01)

within group difference (p<0.01)

Mean ± SEM Mount & Intromission Frequency

Test Week

0 1 2 3 4 5 6 7 8

BASELINE TREATMENT

Ejaculations

Mean ± SEM Ejaculation Frequency

Test Week

0 1 2 3 4 5 6 7 8

BASELINE TREATMENT

FIGURE 19
FIGURE 20

Location of implant tips in E₂-implanted males (closed circles N = 6) and in vehicle-implanted males (open circles, N = 6).
FIGURE 21

Photomicrographs of representative sections from the medial preoptic area (MPO, panels A and B) and the medial amygdala (MEA, panels C and D) from Fadrozole-treated male rats implanted bilaterally in the AMG with either E₂ (A and C) or vehicle (B and D). The photomicrographs in A and C, and B and D, are from the right side of one animal in the E₂-in-AMG group and the right side of one animal in the Vehicle-in-AMG group, respectively. Neuronal labeling by the H222 anti-ER antibody, which is reduced by the presence of estrogen, was abundant in the MPO of both E₂-implanted and vehicle-implanted males and in the MEA of the vehicle-implanted male (A, B, D), but was substantially suppressed in the MEA of the E₂-implanted male (C). This suggests that E₂ was present in the MEA of E₂-implanted, but not of vehicle-implanted, males, and that E₂ in the former had not diffused to the MPO. Scale bar = 300 μm.
FIGURE 21
**TABLE 5**
Mean ± SEM* Behavioral Indices and Numbers of Rats Exhibiting Specific Behaviors by Group

<table>
<thead>
<tr>
<th>Test Week</th>
<th>X ± SEM MF*</th>
<th>X ± SEM IF*</th>
<th>X ± SEM ML*</th>
<th>X ± SEM EL*</th>
<th>X ± SEM PEI*</th>
<th>#Rats: M/I/E (MI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>33.67 ± 5.80</td>
<td>13.00 ± 4.27</td>
<td>152.83 ± 78.10</td>
<td>1293.17 ± 231.72</td>
<td>555.83 ± 248.87</td>
<td>6/6/5 (6)</td>
</tr>
<tr>
<td>-3</td>
<td>32.67 ± 6.71</td>
<td>32.83 ± 4.69</td>
<td>36.17 ± 21.32</td>
<td>885.83 ± 228.62</td>
<td>560.33 ± 248.18</td>
<td>6/6/5 (6)</td>
</tr>
<tr>
<td>-2</td>
<td>28.00 ± 3.82</td>
<td>20.17 ± 5.33</td>
<td>39.67 ± 17.83</td>
<td>963.33 ± 319.09</td>
<td>792.17 ± 319.09</td>
<td>6/6/4 (6)</td>
</tr>
<tr>
<td>-1</td>
<td>42.50 ± 9.86</td>
<td>15.00 ± 4.28</td>
<td>106.67 ± 52.08</td>
<td>1105.50 ± 277.28</td>
<td>1044.83 ± 337.74</td>
<td>6/5/3 (6)</td>
</tr>
<tr>
<td>0</td>
<td>29.00 ± 7.45</td>
<td>16.83 ± 3.81</td>
<td>133.83 ± 105.01</td>
<td>1124.33 ± 105.01</td>
<td>570.50 ± 252.77</td>
<td>6/5/5 (6)</td>
</tr>
<tr>
<td>1</td>
<td>6.50 ± 3.08</td>
<td>5.17 ± 3.29</td>
<td>839.50 ± 339.36</td>
<td>1622.00 ± 178.00</td>
<td>1560.83 ± 239.17</td>
<td>4/2/1 (4)</td>
</tr>
<tr>
<td>2</td>
<td>23.17 ± 6.48</td>
<td>20.50 ± 4.80</td>
<td>149.83 ± 122.47</td>
<td>1615.83 ± 184.17</td>
<td>1555.17 ± 244.83</td>
<td>6/5/1 (6)</td>
</tr>
<tr>
<td>3</td>
<td>37.00 ± 8.68</td>
<td>16.67 ± 6.18</td>
<td>254.50 ± 226.16</td>
<td>1800.00 ± 226.16</td>
<td>1800.00 ± 000.00</td>
<td>6/5/0 (6)</td>
</tr>
<tr>
<td>4</td>
<td>26.00 ± 6.27</td>
<td>18.00 ± 4.35</td>
<td>124.67 ± 94.88</td>
<td>1626.50 ± 122.11</td>
<td>1319.83 ± 303.85</td>
<td>6/6/2 (6)</td>
</tr>
<tr>
<td>5</td>
<td>18.50 ± 7.07</td>
<td>8.17 ± 3.23</td>
<td>519.17 ± 290.58</td>
<td>1738.67 ± 61.33</td>
<td>1573.83 ± 226.17</td>
<td>5/4/1 (5)</td>
</tr>
<tr>
<td>6</td>
<td>25.50 ± 8.29</td>
<td>7.33 ± 4.13</td>
<td>238.83 ± 155.15</td>
<td>1800.00 ± 155.15</td>
<td>1800.00 ± 000.00</td>
<td>6/4/0 (6)</td>
</tr>
<tr>
<td>7</td>
<td>15.17 ± 6.36</td>
<td>11.83 ± 5.36</td>
<td>726.33 ± 348.82</td>
<td>1413.83 ± 352.60</td>
<td>1015.67 ± 000.00</td>
<td>4/4/3 (4)</td>
</tr>
<tr>
<td>8</td>
<td>28.83 ± 3.80</td>
<td>15.67 ± 4.13</td>
<td>275.67 ± 103.80</td>
<td>1616.33 ± 127.76</td>
<td>1354.83 ± 282.79</td>
<td>6/5/2 (6)</td>
</tr>
</tbody>
</table>

*Mean (X) ± standard error of mean (SEM): MF=mount frequency, IF=intromission frequency, ML=mount latency, EL=ejaculation latency, PEI=post-ejaculatory interval

†Number (#) that: M=mounted, I=intromitted, E=ejaculated, MI=mounted or intromitted (were sexually active)
### TABLE 5 (continued)
Mean ± SEM* Behavioral Indices and Numbers of Rats Exhibiting Specific Behaviors by Group

<table>
<thead>
<tr>
<th>Test Week</th>
<th>X ± SEM MF*</th>
<th>X ± SEM IF*</th>
<th>X ± SEM ML*</th>
<th>X ± SEM EL*</th>
<th>X ± SEM PEI*</th>
<th>#Rats: M/I/E (MI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>31.00 ± 4.18</td>
<td>19.67 ± 1.99</td>
<td>71.00 ± 28.73</td>
<td>1400.50 ± 189.09</td>
<td>1065.17 ± 328.84</td>
<td>6/6/3 (6)</td>
</tr>
<tr>
<td>-3</td>
<td>42.33 ± 4.22</td>
<td>29.50 ± 4.23</td>
<td>27.00 ± 10.90</td>
<td>911.67 ± 199.41</td>
<td>531.83 ± 255.65</td>
<td>6/6/5 (6)</td>
</tr>
<tr>
<td>-2</td>
<td>36.00 ± 3.63</td>
<td>23.17 ± 2.56</td>
<td>47.33 ± 9.72</td>
<td>649.33 ± 113.53</td>
<td>306.83 ± 21.88</td>
<td>6/6/6 (6)</td>
</tr>
<tr>
<td>-1</td>
<td>38.67 ± 4.96</td>
<td>24.00 ± 4.26</td>
<td>64.83 ± 34.48</td>
<td>1133.33 ± 249.32</td>
<td>591.83 ± 242.67</td>
<td>6/6/5 (6)</td>
</tr>
<tr>
<td>0</td>
<td>39.17 ± 6.04</td>
<td>26.17 ± 3.70</td>
<td>77.00 ± 45.46</td>
<td>1271.67 ± 334.26</td>
<td>1244.50 ± 352.61</td>
<td>6/6/2 (6)</td>
</tr>
<tr>
<td>1</td>
<td>6.33 ± 5.19</td>
<td>1.17 ± 0.98</td>
<td>1001.83 ± 358.85</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>3/2/0 (3)</td>
</tr>
<tr>
<td>2</td>
<td>18.67 ± 7.88</td>
<td>11.67 ± 4.14</td>
<td>691.67 ± 352.07</td>
<td>1602.17 ± 197.33</td>
<td>1572.67 ± 227.33</td>
<td>4/4/1 (4)</td>
</tr>
<tr>
<td>3</td>
<td>4.50 ± 4.50</td>
<td>1.00 ± 0.98</td>
<td>1591.67 ± 208.33</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>1/1/0 (1)</td>
</tr>
<tr>
<td>4</td>
<td>4.67 ± 4.67</td>
<td>1.83 ± 1.83</td>
<td>1565.33 ± 234.67</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>1/1/0 (1)</td>
</tr>
<tr>
<td>5</td>
<td>10.33 ± 3.53</td>
<td>14.17 ± 4.69</td>
<td>652.00 ± 363.65</td>
<td>1409.83 ± 196.65</td>
<td>841.83 ± 307.71</td>
<td>4/4/4 (4)</td>
</tr>
<tr>
<td>6</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1800.00 ± 311.35</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>0/0/0 (0)</td>
</tr>
<tr>
<td>7</td>
<td>2.67 ± 2.29</td>
<td>0.33 ± 0.33</td>
<td>1309.50 ± 311.35</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>2/1/0 (2)</td>
</tr>
<tr>
<td>8</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>1800.00 ± 0.00</td>
<td>0/0/0 (0)</td>
</tr>
</tbody>
</table>

*Mean (X) ± standard error of mean (SEM): MF=mount frequency, IF=intromission frequency, ML=mount latency, EL=ejaculation latency, PEI=post-ejaculatory interval

†Number (#): M=mounted, I=intromitted, E=ejaculated, MI=mounted or intromitted (were sexually active)