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# Implant of a Selective Estrogen Receptor Alpha Agonist to the Male Rat Medial Preoptic Area Maintains Mating Behavior

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**IMPLANT OF A SELECTIVE ESTROGEN RECEPTOR ALPHA AGONIST TO  
THE MALE RAT MEDIAL PREOPTIC AREA MAINTAINS MATING  
BEHAVIOR**

by

BINIYAM HABTEAB

Under the Direction of Andrew N. Clancy, PhD

**ABSTRACT**

Evidence from knockout studies in male mice and from experiments in male rats, in which expression of the estrogen receptor alpha ( $ER\alpha$ ) gene was inhibited in the medial preoptic area (MPO), suggests that  $ER\alpha$  is important in the control of male rat mating behavior. Therefore, in this experiment, we tested the hypothesis that activation of  $ER\alpha$  in the MPO is sufficient to maintain mating behavior in castrated male rats receiving subcutaneously (s.c.) dihydrotestosterone (DHT), a non-aromatizable androgen. Accordingly, castrated rats treated with DHT s.c. received MPO implants of either: (i) propyl-pyrazole-triol (PPT) (Stauffer, *et al* 2000; Katzenellenbogen, *et al* 2000), a selective  $ER\alpha$  agonist, (ii)  $E_2$  (positive controls) or (iii) cholesterol (negative controls) and sexual behavior was monitored. PPT was as effective as  $E_2$  at maintaining mating behavior suggesting that, in the MPO,  $ER\alpha$  is sufficient to mediate responses to  $E_2$  that underlie male rat mating behavior.

**INDEX WORDS:** Medial preoptic area, Estrogen receptor, Estradiol, Dihydrotestosterone, propyl-pyrazole-triol (PPT), Cholesterol, Sexual behavior

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**BINIYAM HABTEAB**

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2007

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2007

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**LIST OF ABBREVIATIONS AND SYMBOLS**

AD	Alzheimer's disease
AH	anterior hypothalamus
NOVA	analysis of variance
ApoE2	apolipoprotein E2
ApoE3	apolipoprotein E3
ApoE4	apolipoprotein E4
AR	Androgen receptors
AS-ODN	Antisense oligodeoxynucleotide)
ATD	1,4, 6-androstatriene-3,17-dione
BST	Bed nucleus of the stria terminalis
cAMP	cyclic adenosine mono phosphate
C1	carbon one
C2	carbon two
C3	carbon three
C4	carbon four
C5	carbon five
N1	nitrogen one
DAG	diacylglycerol
DA	dopamine
D <sub>1</sub>	dopamine receptors 1
D <sub>2</sub>	dopamine receptors 2

Df	Degrees of freedom
DHT	Dihydrotestosterone
DHTP	Dihydrotestosterone propionate
DPN	diarylpropionitrile
E <sub>2</sub>	Estradiol
E <sub>2</sub> .BSA	E <sub>2</sub> -bovine serum albumin
EB	Estradiol benzoate
EF	Ejaculation frequency
EL	Ejaculation latency
ER	Estrogen receptor
ER $\alpha$	Estrogen receptor alpha
ER $\beta$	Estrogen receptor beta
ER $\alpha$ KO	Estrogen receptor alpha knockout
ER $\beta$ KO	Estrogen receptor beta knockout
$\alpha\beta$ ERKO	Alpha and beta estrogen receptor knockout
G6PDH	glucose-6-phosphate dehydrogenase
HB-EGF	heparin-binding epidermal growth factor
HR	Hit rate
i.d.	inside diameter
IF	Intromission frequency
IMI	inter-mount interval
LDL	low-density lipoprotein

L-MMA	N-monomethyl-L-arginine
LPM	Liter per minute
M	Mean
mm	Millimeter
MEA	Medial amygdale
MF	Mount frequency
ML	Mount latency
MPO	Medial preoptic area
NOS	nitro oxide synthase
nNOS	neuronal nitro oxide synthase
o.d.	outside diameter
p	Probability associated with the occurrence under the null hypothesis a
PEI	Post ejaculatory intervals
PLC	phospholipase C
PPD	propylpyrazole diol
PPT	Propyl-pyrazole-triol
SEM	Standard error of mean
s.c.	Subcutaneously
t	Computed value of t test
T	Testosterone
TP	Testosterone propionate
<	Less than

= Equal to

**IMPLANTS OF A SELECTIVE ESTROGEN RECEPTOR ALPHA AGONIST TO  
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**INTRODUCTION**

The medial preoptic area (MPO) is located in the forebrain in the rostral hypothalamus and plays a key role in the expression of male mating behavior (Simerly, 2002). Male rats with MPO lesion showed highly suppressed levels of mounting and intromission and are completely unable to ejaculate (Liu *et al.*, 1997), suggesting that the MPO is necessary for ejaculation. Similarly, lesions of the male rat medial amygdala (MEA) also compromise mating behavior by reducing mounting and intromission, consistent with the possibility that motivation and sexual arousal are decreased (Kondo, 1992). Both testosterone (T) and its androgenic (DHT) and estrogenic (E<sub>2</sub>) metabolites act in the MPO and both types of metabolites are necessary for mating behavior to occur (Morali, 1986; Christensen *et al.*, 1986). Implant of testosterone propionate (TP) in the MPO maintains copulatory behavior in castrated male rats including mounting, intromission, and ejaculation (Morali, 1986), as do MPO implants of estradiol (E<sub>2</sub>) (if an androgen source is present) (Michael *et al.*, 1973; Baum *et al.*, 1973; McGinnis *et al.* 1989) and dihydrotestosterone (DHT) (if an estrogen source is present) (McGinnis, 1989; Naftolin *et al.*, 1972; Baum, 2003). Under normal circumstances, T is converted into E<sub>2</sub> by aromatase in the brain (Powers *et al.*, 1987; Naftolin *et al.*, 1972) and aromatase

mRNA is expressed in the MPO (Roselli *et al.*, 2000). Testosterone is also converted in the testis or the brain into DHT by 5 alpha-reductase (Whalen *et al.*, 1985; Martini, 1982; Massa, *et al* 1981). Castrated rats treated with either E<sub>2</sub> alone or DHT alone fail to express the full repertoire of copulatory behavior (Wallis *et al.*, 1975; Per Sodersten, 1973). However, castrated rats displayed normal mating behavior to ejaculation after being treated with a combination of E<sub>2</sub> and DHT and their mating behavior was equal to that of rats treated with T (Michael *et al.*, 1973; Baum *et al.*, 1973). Implants of 1,4,6-androstatriene-3,17-dione (ATD), a steroidal aromatase inhibitor, plus TP in the MPO hinder copulatory behaviors, but implant of ATD with estradiol benzoate (EB) in the MPO promote copulatory behavior (Watson *et al.*, 1989). It is worth mentioning, however, that ATD may compete for receptor binding with steroids (Kaplan *et al.*, 1989). Furthermore, gonadectomized male rats treated s.c. with the combination of T and fadrozole, a non-steroidal aromatase inhibitor that blocks the conversion of testosterone to E<sub>2</sub>, failed to show copulatory behavior and exogenous E<sub>2</sub> partially reversed this (Bonsall *et al.*, 1992; Roselli, *et al* 2003; Vagell *et al.*, 1997). Similarly, infusion of fadrozole to the MPO of gonadally intact male rats inhibited mating (Clancy, 1995), whereas gonadally intact male rats treated with fadrozole s.c. together with E<sub>2</sub> implants in the MPO displayed normal copulatory behavior (Clancy, 2000). Collectively, these findings suggest that E<sub>2</sub> acts in the MPO and that this action is necessary to maintain sexual behavior in male rats (Clancy *et al.*, 2000; Clancy *et al.*, 1995). The rat MPO

contains more than one type of estrogen-sensitive neuron; some express estrogen receptor alpha ( $ER\alpha$ ) (Shughrue et al., 1997; Kumar *et al.*, 2006) others express estrogen receptor beta ( $ER\beta$ ) (Krege et al., 1998; Greco, 1998) some express both  $ER\alpha$  and  $ER\beta$  (Abraham *et al.*, 2004; Nomura *et al.*, 2003), others express an ER in the membrane that is G-protein-coupled (Sinchak *et al.*, 2001). After treatment with TP and dihydrotestosterone propionate (DHTP), castrated male mice with estrogen receptor  $\alpha$  knockout ( $ER\alpha$ KO) were able to mount and intromit, but were unable to ejaculate, suggesting a role for  $ER\alpha$  in control of ejaculation (Ogawa *et al.*, 1998; Scordalakes *et al.*, 2002). In contrast to  $ER\alpha$ KO, estrogen receptor  $\beta$  knockout ( $ER\beta$ KO) castrated male mice showed relatively normal sexual behavior, similar to that of castrated wild type mice treated with TP and DHTP (Ogawa *et al.*, 1999) suggesting  $ER\beta$  is not necessary. However, mice with combined  $\alpha\beta$ ERKO failed to mate at all (Ogawa *et al.*, 2000), so both  $ER\alpha$  and  $ER\beta$  may play a role in the display of mating behavior. Placement of an antisense oligodeoxynucleotide (AS-ODN) complementary to  $ER\alpha$  mRNA into the MPO inhibits the expression of  $ER\alpha$  and reduces levels of mounting, intromission and ejaculation, unlike control rats that had been infused in the MPO with saline or received AS-ODN in the MEA, which mated normally to ejaculation (Paisley *et al.*, 2006). This suggests that  $E_2$  acts differently in different brain regions,  $ER\alpha$  in MPO is necessary for male rat mating behavior, and  $ER\alpha$  is not necessary in the MEA (Paisley *et al.*, 2006).

Collectively, the evidence from these studies suggest a major role for  $ER\alpha$  in the

control of male rat copulatory behavior and a lesser role for ER $\beta$ , but additional research is required to determine the exact roles of these receptor subtypes in the control of mating and to determine where in the brain each subtype may play a role. We hypothesize that ER $\alpha$  in the MPO is sufficient to promote copulatory behavior in castrated male rats treated s.c. with DHT. MPO implants of PPT were used rather than systemic administration of PPT because we hoped to test where in the brain PPT might act. Therefore, we compared the effects on ejaculation of implants to the MPO of either E<sub>2</sub> or propyl-pyrazole triol (PPT) (Stauffer *et al.*, 2000), a selective estrogen receptor  $\alpha$  agonist, in sexually experienced castrated male rats receiving DHT s.c., which supplied the whole brain with a necessary non-aromatizable androgen source. We predicted that PPT would be as effective as E<sub>2</sub> at maintaining male rat mating behavior, demonstrating the behavioral relevance and sufficiency of ER $\alpha$  activation in the MPO.

## MATERIALS AND METHODS

### *Animals*

Thirty Sprague Dawley male rats and fifteen female rats were allowed free access of food and water and kept in cages (22 X 44 X 50 cm) in the Georgia State University vivarium, at a temperature between 20<sup>0</sup>-23<sup>0</sup> Centigrade and humidity of 43-56%. The rats were maintained on 14:10 hours reverse light: dark cycle (light off at 9:30 AM EST). Male rats lived two per cage until surgery after which they are housed singly and

females were housed two per cage throughout the study. Animal care was in accordance with humane standards (NIH publ. No. 85-23, revised 1985) and all procedures involving animals were authorized by the Georgia State University IACUC.

### ***Female rats surgical procedures***

Stimules females were anesthetized with isoflurane gas in a chamber (5% gas and 5 LPM oxygen exchange rate) and transferred to a nosecone (2-3% gas and 3 LPM oxygen). Ovaries were removed through an abdominal incision and rats were implanted in the scapular region s.c with a Silastic capsule (6mm length, 1.981 mm i.d. x 3.175 mm o.d.) containing crystalline E<sub>2</sub> (Clancy *et al.*, 1995). Females were allowed at least five days to recover from surgery before they were paired with male rats to measure sexual behavior in the males. Four hours prior to each weekly behavioral test, females were injected s.c. with 1 mg progesterone in 0.2 ml of sesame oil to induce sexual receptivity.

### ***Male behavior screening, surgical procedures, and testing***

Thirty male rats were screened once weekly for three consecutive weeks for the display of sexual behavior prior to being selected in the experiment. Tests for sexual behavior were conducted by blind observers once a week, 30 minutes per test. Sex testing took place under red light illumination during the dark phase of the light: dark cycle. The recorded behaviors included: mount frequency (MF): number of mounts with no penetration, intromission frequency (IF): numbers of intromission with penetration, ejaculation frequency (EF): numbers of ejaculation, mount latency (ML): time from test

start until the first mount or intromission or 1800 seconds if mounts did not occur, ejaculation latency (EL): time from first mount or intromission until the first ejaculation or 1800 seconds if ejaculation did not occur, post ejaculatory interval (PEI): the time between the first ejaculation and the next mount or intromission or 1800 seconds if ejaculation did not occur, hit rate (HR): the percentage of mounts accompanied by intromission and inter-mount interval (IMI): average time between consecutive mounts or intromissions during the first mount bout or 1800 for non-ejaculations. (Clancy, A. N., Zumpe, D., and Michael, R. P. 2000; Liu, Y., Salamone, J. D., and Sachs, B. D. 1997). Thereafter, male rats were assigned into three matched groups based on ejaculation frequency: (i) PPT (n=7), (ii) E<sub>2</sub> (n=8) or (iii) cholesterol (n=7). All three groups were implanted in the MPO with the appropriate drug. Specifically, males were anesthetized with isoflurane gas in a chamber (5% gas and 5 LPM oxygen exchange) and transferred to a nosecone (2-3% gas and 3 LPM oxygen exchange). Anesthetized rats were castrated through abdominal incisions and received s.c. a Silastic capsule containing crystalline DHT (15 mm length, 1.981 mm i.d. and 3.175 mm o.d.) in the scapular region. They were then placed in stereotaxic instrument and implanted bilaterally with 22 gauge guide cannulae aimed at the MPO. (Level skull coordination: anterior posterior = -0.5 mm, medial lateral = ± 0.75 mm, dorsal ventral = - 8.0 mm Swanson et al., 1998). 28 gauge inner cannulae, extended 1 mm below guide cannulae. These were tamped in either E<sub>2</sub>, PPT or cholesterol to deliver the drug into the MPO. Thereafter, rats were allowed at

least five days to recover from surgery and postoperative behavioral testing began. Sterile inner cannulae were replaced 48 hours before and 24 hours after each behavior test under brief isoflurane anesthesia. Tips of cannulae were examined microscopically after they were removed from the brain to insure drug was present and all cannulae passed this inspection.

### ***Histology***

After completion of behavior testing, cannulae placements were confirmed by histology. Males were euthanized with lethal doses of Nembutal, (sodium pentobarbital 12 ml/kg, i.p, Abbott Laboratories), and transcardially perfused with saline, followed by 300 ml of fixative (4% paraformaldehyde, in 0.1 M phosphate buffer). Brains were then immersed in fixative for at least 24 hours, transferred to 30% sucrose in 0.1 M phosphate buffer for at least two days and cut coronally into 40  $\mu\text{m}$  sections on a freezing microtome. Sections were stained with thionin and locations of the cannulae tips were mapped as black spots onto standard atlas plates.

### ***Statistical analyses***

Repeated measures (groups x trials) analysis of variance (ANOVA) was used for statistical comparisons among the groups (Kirk, 1968). Follow-up post-hoc analysis (Tukey honesty significant differences test,  $\alpha = 0.05$ ) was used to identify significant group differences. Paired t-tests were used to compare within group changes in each group between the third pre-operative behavior test (terminal pre-operative performance)

and the fourth post-operative behavior test (terminal post-operative performance). Two tailed probabilities are reported in all cases.

## RESULTS

### *Behavior*

Both estradiol and PPT implants to the MPO maintained the mating behavior of sexually experienced, castrated male rats receiving DHT s.c. but cholesterol MPO implants were ineffective. Thus, the two groups receiving either estradiol or PPT MPO implants mated vigorously and were statistically indistinguishable from each other on all measures of copulatory behavior during both the preoperative and postoperative periods; moreover, sexual behavior did not change significantly in either of these two groups in the periods before and after surgery. In contrast, sexually experienced, castrated, DHT-treated males that received MPO cholesterol implants virtually ceased mating during the postoperative period and was significantly lower than that of either of the other two groups on all measures of copulatory behavior, although, during the preoperative period, they had copulated robustly and did not differ significantly on any behavioral measure from the rats in the other two groups. Moreover, all measures of sexual behavior declined significantly in the cholesterol MPO group during the period after surgery relative to preoperative levels.

During the postoperative period (FIGURE 1), the groups differed significantly from each other on mount frequency ( $F_{2,19} = 10.23, p < 0.001$ ), intromission frequency ( $F_{2,19} = 10.80, p < 0.001$ ), ejaculation frequency ( $F_{2,19} = 10.98, p < 0.001$ ) and hit rate

( $F_{2,19} = 9.31, p < 0.002$ ). Moreover (FIGURE 2), during the postoperative period, the groups also differed significantly from each other on mount latency ( $F_{2,19} = 13.80, p < 0.0001$ ), ejaculation latency ( $F_{2,19} = 6.66, p < 0.007$ ), inter-mount interval ( $F_{2,19} = 6.86, p < 0.006$ ) and the post-ejaculatory interval ( $F_{2,19} = 8.12, p < 0.003$ ). No other statistically significant main effects or interactions were observed during the postoperative period. Follow-up post-hoc analysis of behavior during the postoperative period revealed no significant differences on any test day between the estradiol and PPT groups on any of the eight behavioral measures, however, mount frequency in the cholesterol group was significantly lower than that in each of the other two groups on the second ( $p < 0.05$ ) and third ( $p < 0.05$ ) days; on intromission frequency, the cholesterol group was significantly lower ( $p < 0.05$ ) than the estradiol group on all four trials and lower than the PPT group on the second and fourth trials ( $p < 0.05$ , respectively); and on hit rate, the cholesterol group was significantly lower ( $p < 0.05$ ) than the PPT group on the fourth day (FIGURE 1). Similarly (FIGURE 2), during postoperative trials, the cholesterol group exhibited significantly longer mount latencies than the estradiol group on the second, third and fourth days and PPT group on the second and fourth days ( $p < 0.05$ , respectively); on ejaculation latency, the cholesterol group was significantly ( $p < 0.05$ ) higher than the estradiol group on the second day; and with respect to the inter-mount interval and the post-ejaculatory interval on the second postoperative trial, the cholesterol group showed significantly longer latencies than both the estradiol and PPT groups ( $p < 0.05$ , respectively).

There were no significant group differences on any of the eight measures of copulatory behavior during the preoperative trials, nor were there any significant interactions. Significant behavioral differences emerged, however, as the days of testing progressed during the preoperative period. Specifically, mount frequency scores ( $F_{2,38} = 24.76$ ,  $p > 0.0001$ ) and intromission frequency scores ( $F_{2,38} = 22.93$ ,  $p > 0.0001$ ) improved significantly as preoperative testing advanced (FIGURE 1), whereas mount latency scores decreased significantly ( $F_{2,38} = 12.90$ ,  $p > 0.0001$ ) during the preoperative period.

Changes in sexual behavior within each group were examined by comparing the performances on the last preoperative testing day and the last postoperative testing day. There were no significant changes on any of the eight indices of copulatory behavior in the groups that received MPO implants of either estradiol or PPT, however, on all eight measures of mating, sexual behavior deteriorated significantly during the postoperative period in the group implanted with cholesterol to the MPO. Specifically, in the cholesterol group, mount frequency ( $t = 8.15$ ,  $df = 6$ ,  $p < 0.0002$ ), intromission frequency ( $t = 6.05$ ,  $df = 6$ ,  $p < 0.0009$ ), ejaculation frequency ( $t = 4.80$ ,  $df = 6$ ,  $p < 0.003$ ) and hit rate ( $t = 2.84$ ,  $df = 6$ ,  $p < 0.03$ ) were significantly depressed on the last postoperative test compared with the last preoperative day (FIGURE 1), whereas mount latency ( $t = 3.99$ ,  $df = 6$ ,  $p < 0.007$ ), ejaculation latency ( $t = 2.88$ ,  $df = 6$ ,  $p < 0.03$ ), inter-mount interval ( $t = 3.94$ ,  $df = 6$ ,  $p < 0.007$ ) and the post-ejaculatory interval ( $t = 4.73$ ,  $df = 6$ ,  $p < 0.003$ ) were significantly lengthened on the last postoperative test relative to the last preoperative day (FIGURE 2).

### *Histology*

Locations of the cannulae tips were mapped as black spots onto standard atlas plates; all cannulae tips were located in the MPO or anterior hypothalamus (FIGURE 3).

FIGURE 1: Mount frequency, intromission frequency, ejaculation frequency, and hit rate percentage.

Mean  $\pm$  standard error of the mean (SEM) mount frequency, intromission frequency, ejaculation frequency, and hit rate in weekly, 30 min sex tests. During the post operative but not preoperative periods the groups differed significantly on each of these behavioral measure (See text for F values).

\*: Between-group comparison: cholesterol differs significantly from E<sub>2</sub>,  $p < 0.05$ .

+: Between-group comparison: cholesterol differs from PPT,  $p < 0.05$ .

X: Cholesterol within group comparison,  $p < 0.05$ .

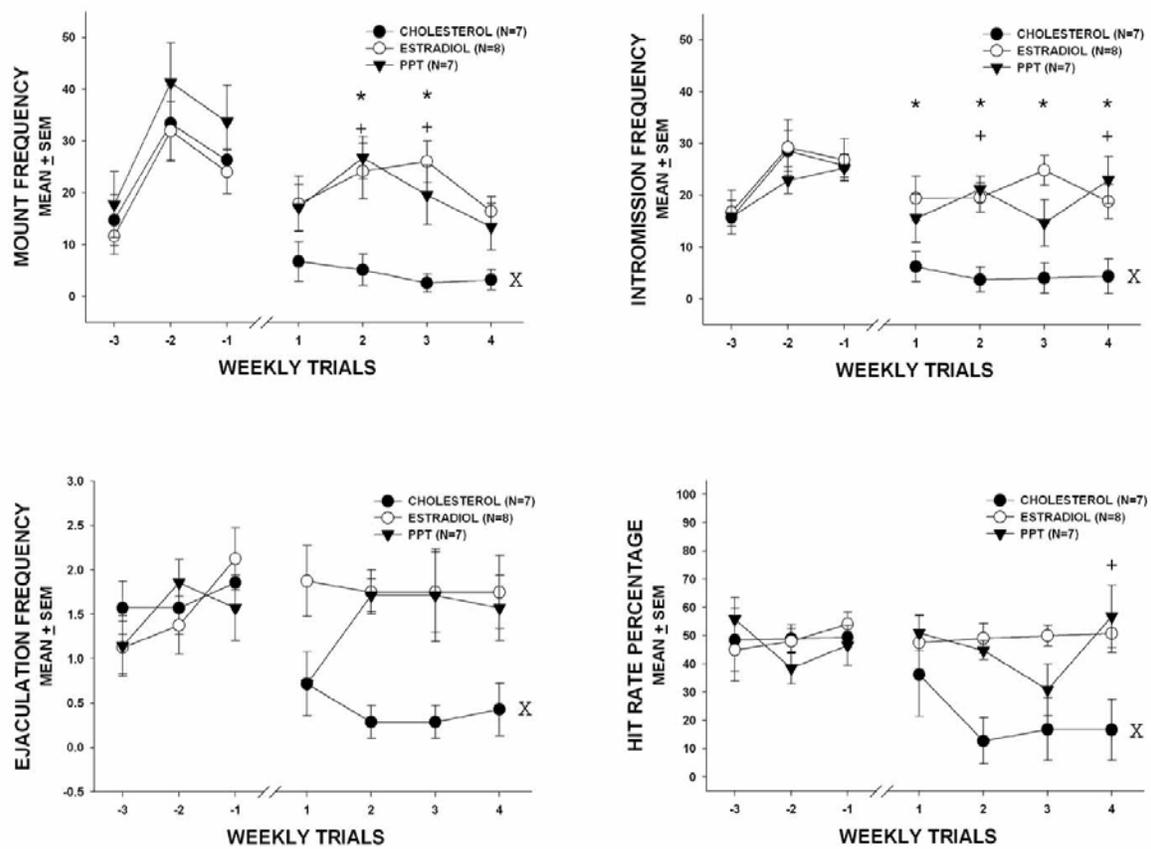


FIGURE 1

FIGURE 2: Mount latency, inter-mount interval, ejaculatory latency, and post ejaculatory interval.

In seconds, mean  $\pm$  SEM mount latency, inter-mount interval, ejaculatory latency, and post ejaculatory interval in weekly, 30 min sex tests. During the post operative but not preoperative periods the groups were differed significantly on each of these behavioral measure (See text for F values).

\*: Between-group comparison: cholesterol differs significantly from E<sub>2</sub>,  $p < 0.05$ .

+: Between-group comparison: cholesterol differs from PPT,  $p < 0.05$ .

X: Cholesterol Within group comparison,  $p < 0.05$ .

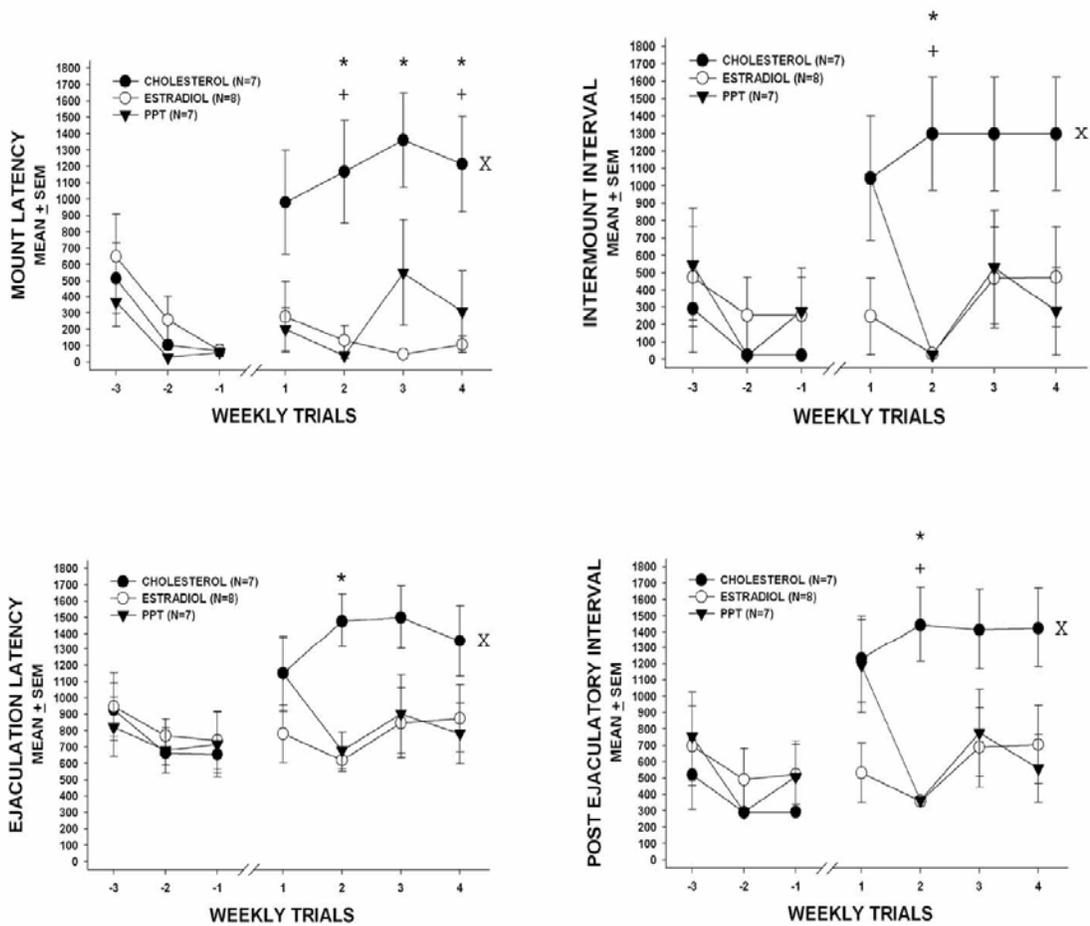


FIGURE 2

FIGURE 3: Cannulae Placement.

The cannulae tips were located in the MPO or anterior hypothalamus (AH). The distribution of cannulae tips in the MPO was mapped on a standard series of coronal atlas plates through the MPO and anterior hypothalamus. Dots represent locations of cannulae tips from each animal.

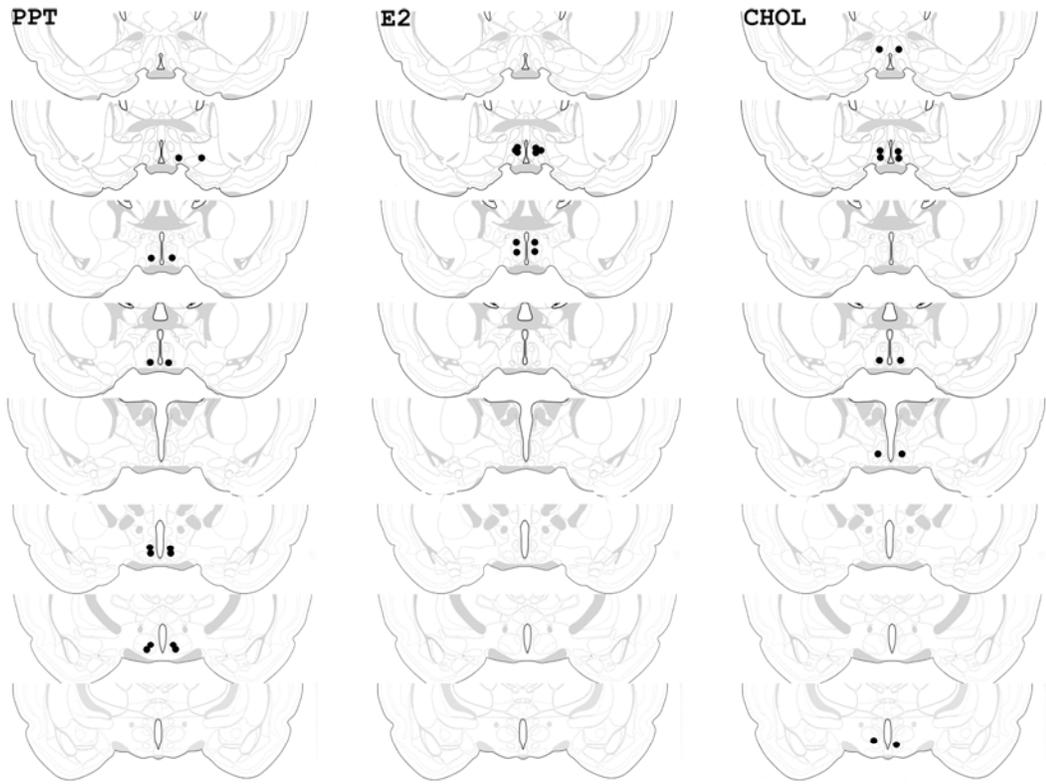


FIGURE 3

## DISCUSSION

In this study, we compared the copulatory behavior of sexually experienced, castrated rats treated with DHT s.c that received MPO implants of either PPT, an estrogen receptor  $\alpha$  agonist,  $E_2$  or cholesterol. PPT treated rats displayed comparable levels of mating behavior to those treated with  $E_2$ , whereas mating virtually ceased in cholesterol treated animals as shown in other studies (Rosenblatt *et al.*, 1998). These findings support the hypothesis that ER $\alpha$  in the MPO is sufficient to promote mating behavior in castrated male rats treated s.c. with DHT. PPT is thought to activate ER $\alpha$  only whereas  $E_2$  activates both ER $\alpha$  and ER $\beta$  yet rats treated with PPT mounted, intromited and ejaculated at rates that were statistically indistinguishable from those treated with  $E_2$ . In the cholesterol group, on the other hand, presumably neither ER $\alpha$  nor ER $\beta$  were activated and these rats virtually ceased mating.

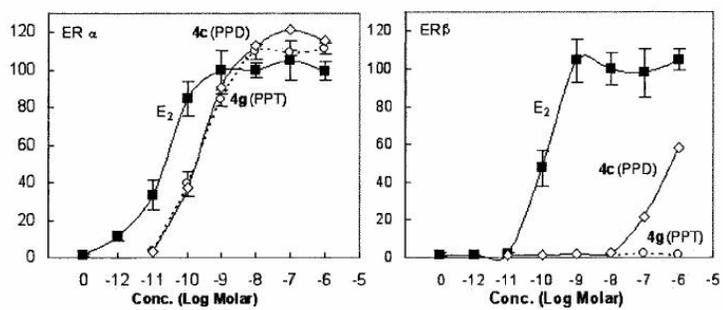
A critical region of the brain for male copulation control is the MPO, as much prior research has shown (Clancy *et al.*, 2000; Clancy, et al., 1995; Bonsall et al., 1992; Morali, 1986). Steroid sensitive neurons in the MPO are activated by mating (Greco *et al.*, 1998), including those expressing androgen receptors (Greco *et al.*, 1999), ER $\alpha$  and ER $\beta$  (Shughrue et al., 1997; Kumar *et al.*, 2006; Krege et al., 1998; Greco, 1998) and there is much evidence suggesting that ER $\alpha$  is the behaviorally relevant ER (Ogawa *et al.*, 1998; Scordalakes *et al.*, 2002; Paisley *et al.*, 2006). The present results demonstrate that activation of ER $\alpha$  in the MPO is sufficient to promote copulatory behavior in castrated DHT treated male rats. Specifically implants of the MPO with PPT, a selective

ER $\alpha$  agonist, supported mating equally as well as E<sub>2</sub>, which stimulates both estrogen receptor  $\alpha$  and  $\beta$ .

In human cell culture, PPT binds ER $\alpha$  with the same affinity as E<sub>2</sub> binds ER $\alpha$  and has zero cross reactivity with ER $\beta$  (Stauffer *et al.*, 2000) (FIGURE-4).

FIGURE 4: PPT binds to ER $\alpha$  but not ER  $\beta$ .

“Transcription activation by ER $\alpha$  (left) and ER $\beta$  (right) in response to pyrazole 4c (PPD) and 4g (PPT). Human endometrial cancer (HEC-1) cells were transfected with expression vectors for ER $\alpha$  or ER $\beta$  and an (ERE) 3-pS-CAT reporter gene and were treated with the indicated concentrations of ligand for 24 h. CAT activity was normalized for  $\beta$ -galactosidase activity from an internal control plasmid. Values are expressed as a percent of the ER $\alpha$  or ER $\beta$  response with 2 nM E<sub>2</sub>, which is set at 100%” (Stauffer *et al.*, 2000).



Stauffer *et al.*, 2000

FIGURE 4

Ligands such as PPT or estradiol bind selectively to receptors depending on the ligand binding pocket, cofactors, estrogen response elements and their promoters and other factors (Wright *et al.*, 2006). For example, ER $\alpha$  has in its ligand binding pocket Met 421 and ER $\beta$  has Ile 373; the ER $\alpha$  ligand binding pocket is occupied by Leu384, on the other hand, ER $\beta$  has Met 336 (Wright *et al.*, 2006; Katzenellenbogen *et al.*, 2000). PPT is formed from pyrazole attached at C3 and C5 with phenol, N1 phenyl and C4 with propyl group (Wright *et al.*, 2006). The hydroxyl portion of the phenol group at C3 and C5 have no charge and can react with the hydrophilic side chain of other compounds in order to form hydrogen bonds (Stauffer *et al.*, 2000). Both the nitrogen groups at C1 and C2 also have no charge and they can form a hydrogen bond with hydrophilic side chain of the receptor proteins or water (Stauffer *et al.*, 2000).

*In vitro* experiments demonstrate that ER $\alpha$ , but not ER $\beta$ , bind to PPT. Relative binding affinity is measured after replacing the R (alkyl group) with different alkyl groups and the X (halogen group) is replaced with hydrogen or hydroxy groups (Stauffer *et al.*, 2000). These competitive radiometric binding assays were measured in human ER $\alpha$  and ER $\beta$  (Stauffer *et al.*, 2000). The assay showed that PPT has greater affinity toward ER $\alpha$  by 410 fold than to ER $\beta$  (Stauffer *et al.*, 2000). The ability of PPT to activate transcription of ER $\alpha$  or ER $\beta$  genes were demonstrated in human endometrial cancer cells (Stauffer *et al.*, 2000). The potency selectivity between ER $\alpha$ /ER $\beta$  to PPT indicates that PPT binds to ER $\alpha$  with greater potency than ER $\beta$  (Stauffer *et al.*, 2000).

PPT has been used in various experiments as an ER $\alpha$  agonist. For instance, PPT activates ER $\alpha$ , whereas diarylpropionitrile (DPN) activates ER $\beta$ , in hippocampus *in vitro*

and *in vivo* (Wang *et al.*, 2006). Activation of ER $\alpha$  via PPT promotes the formation of apolipoprotein E4 (ApoE4) which is associated with Alzheimer's disease (AD), triggering an increase in low-density lipoprotein (LDL), and neurodegenerative disease (Harris *et al.*, 2003). On the other hand, the ER $\beta$  agonist, DPN, inhibits ApoE4 and activates apolipoprotein E2 (ApoE2) and apolipoprotein E3 (ApoE3), which is linked to a reduced risk of AD (Wang *et al.*, 2006). Similar observations were also seen in immature mice that had been injected with PPT; PPT increased uterus weight (Frasor *et al.*, 2003). Collectively these studies demonstrate that PPT activates at least three different genes that include complement component 3 (C3), lactoferrin, and glucose-6-phosphate dehydrogenase (G6PDH) in the uterus (Frasor *et al.*, 2003).

Our new finding extends previous reports showing that blockade of ER $\alpha$  expression in the MPO impairs mating behavior (Paisley *et al.*, 2006). Specifically infusion of the MPO with an antisense oligodeoxynucleotide sequence complementary to ER $\alpha$  mRNA reduced expression of ER $\alpha$  and sexual behavior in male rats (Paisley *et al.*, 2006), suggesting a role for ER $\alpha$  in control of mating behavior. Similarly, male ER $\alpha$  knockout mice also show reduced mating behavior (Ogawa *et al.*, 1998; Scordalakes *et al.*, 2002). The present findings complement these earlier reports by demonstrating that a selective ER $\alpha$  agonist drug maintains behavior equally as well as E<sub>2</sub>, which suggests the behaviorally relevant ER in the male rat MPO is ER $\alpha$  with respect to mating behavior.

Each behavior we observed, MF, IF, EF, ML, EL, PEI, HR and IMI, is maintained by E<sub>2</sub> activation of ER in the MPO and specifically by ER $\alpha$ , since PPT MPO implants

were as effective as E<sub>2</sub> MPO implants at promoting mating in gonadomatized male rats treated with exogenous DHT, but cholesterol MPO implants were ineffective.

It is pertinent that T is converted into E<sub>2</sub> through aromatization (Naftolin *et al.*, 1972; Powers *et al.*, 1987), and DHT is formed from T through the action of 5- $\alpha$  reductase (Martini, 1982; Massa, *et al.*, 1981; Whalen *et al.*, 1985). Exogenous T in physiological concentrations is sufficient to promote copulatory behavior in castrated male rats, and combined treatment with E<sub>2</sub> and DHT, each in physiological concentration, is as effective as T in promoting sexual behavior (Michael *et al.*, 1973; Baum *et al.*, 1973; McGinnis *et al.* 1989; McGinnis, 1989; Naftolin *et al.*, 1972; Baum, 2003). In contrast, sexual behavior is poorly maintained by either E<sub>2</sub> alone or DHT alone except if substantially in higher doses in the pharmacological range are administered (McGinnis, 1989). It is noteworthy that T must be converted into E<sub>2</sub> and DHT to maintain sexual behavior in castrated rats and if this conversion of T to E<sub>2</sub> is blocked, then sexual behavior is depressed (Naftolin *et al.*, 1972; Baum, 2003; Bonsall *et al.*, 1992). E<sub>2</sub> acts in the brain by binding to estrogen receptors whereas T and DHT bind to androgen receptors. Both estrogen receptors and androgen receptors are found in different regions of the brain, including the MPO, supraoptic nucleus, ventral zone of medial parvicellular part of the paraventricular nucleus of hypothalamus and lateral cerebellar region (Simerly *et al.*, 1990). Since, both ER and AR reside within the MPO (Handa *et al.*, 1996), both ER and AR containing neurons have the potential to mediate male rat mating.

There are at least two ways that E<sub>2</sub> may affect its targets, either by activating the genome or via a non-genomic pathway; many questions remain concerning how estrogen

acts in the MPO. One neuronally mediated estrogen dependent response that has received considerable attention is the estrogen activation, via ER $\alpha$ , of MPO dopamine circuits (Scordalakes *et al.*, 2002; Putnam *et al.*, 2005). It has been proposed that E<sub>2</sub> activation of ER $\alpha$  enhances the formation of neuronal nitro oxide synthase (nNOS) (Scordalakes *et al.*, 2002) which converts L-arginine into citrulline and nitric oxide (NO) (Palmer *et al.*, 1988). NO, in turn, triggers the release of dopamine (DA) (Scordalakes *et al.*, 2002;) in the MPO. This increase in dopamine in the MPO elicits erection and ejaculation (Hull *et al.*, 1992; Hull *et al.*, 1989; Dominguez *et al.*, 2004; Hull *et al.*, 1999; Putnam *et al.*, 2005). There is also a negative feed back mechanism whereby elevated levels of NO inhibit aromatase, which limits further production of E<sub>2</sub> from T (Snyder *et al.*, 1996). Castrated animals tend to have decreased nNOS but exogenous administration of T or E<sub>2</sub> reverses this effect of castration and nNOS subsequently increases (Du, *et al.*, 1999; Putnam *et al.*, 2005). Furthermore, castration also decreases the release of DA from the MPO and this is due to lack of NO in the MPO (Du, *et al.*, 1998). Immunocytochemical studies have demonstrated the coexpression of AR, ER $\alpha$  and nNOS in the MPO (Sota *et al.*, 2005). Implantation of the nitro oxide synthase (NOS) inhibitor, N-monomethyl-L-arginine (L-NMMA), blocked the expression of NO in the MPO and reduced the mount rate. Conversely, administration of L-arginine, a NO precursor, in the MPO promoted an increase of mounting in male rats (Sota *et al.*, 1998), whereas blocking the synthesis of NOS in an intact rat inhibits the formation of DA and lowered mating behavior (Hull *et al.*, 1997).

Castrated male rat failed to achieve erection following electrical stimulation of MPO, but implant of T in the MPO reinstated erection (Suzuki *et al.*, 2007; Giuliano, 1997). This suggests that MPO plays a role in an erection in response to T (Suzuki *et al.*, 2007). Stimulation of dopamine receptor 1 (D<sub>1</sub>) in the MPO enhanced erection by activating parasympathetic pathways, and dopamine receptor 2 (D<sub>2</sub>) in the MPO operating through a sympathetic pathway to elicit ejaculation (Hull *et al.*, 1992; Hull *et al.*, 1989; Dominguez *et al.*, 2004).

In the conclusion, there is much evidence suggesting that ER $\alpha$  is the behaviorally relevant ER for mating. We demonstrated that activation of ER $\alpha$  in the MPO is sufficient to promote copulatory behavior in castrated DHT treated male rats. We implanted PPT, a selective ER $\alpha$  agonist, in MPO which maintained mating behavior equally as well as E<sub>2</sub>, (which stimulates both ER $\alpha$  and ER $\beta$ ). Sexual behavior data gathered from this PPT study suggests that ER $\alpha$  is the ER in the MPO that is sufficient for display of mating behavior. Further investigation is required to identify whether MPO ER acts as a membrane receptor or whether it acts as transcription factor in promoting sexual behaviors. More studies will be required to determine other sites in the brain where PPT may act and to test if its effects in the MPO are specific by incorporating anatomical controls and by examining the MPO more closely to see if PPT has selective effects in different sub regions of the MPO. However, recent finding in our laboratory indicated that castrated, DHT treated male rats implanted with E<sub>2</sub> conjugated to bovine serum albumin (BSA) in the MPO were able to mate normally (Huddleston *et al.*, 2006, Personal communication). This raises the possibility that ER $\alpha$  associated with the plasma

membrane of MPO neurons may mediate the responses to E<sub>2</sub> that underlie male rat capulatory behavior.

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