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Antisense Oligodeoxynucleotides for Estrogen Receptor-β and α Attenuate Estradiol's Modulation of Affective and Sexual Behavior, Respectively

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Estradiol (E_2) modulates affective and socio-sexual behavior of female rodents. E_2's functional effects may involve actions through α and β isoforms of estrogen receptor (ERs). The importance of E_2's actions at these isoforms for anxiety (open field, elevated plus maze), depression (forced swim test), and sexual behavior (lordosis) was investigated using an antisense oligonucleotide (AS-ODN) strategy. If ERβ is required for anti-anxiety and antidepressant-like effects, and ERα is required for sexual receptivity, of E_2, then intracerebroventricular administration of AS-ODNs against these ERs should attenuate these effects and reduce immunoreactivity of ERs in brain regions that mediate these behaviors, such as the hippocampus and ventral medial hypothalamus (VMH). Ovariectomized rats were primed with 17β-E_2 (10 μg) 48 h before testing (hour 0). At hours 0, 24, and 47.5, rats were infused with saline vehicle, scrambled control AS-ODNs, or AS-ODNs targeted against ERα and/or ERβ, and were tested at hour 48. Rats infused with ERβ AS-ODNs, alone, or with ERα AS-ODNs had significantly decreased open field central entries, decreased plus maze open arm time and entries, increased time spent immobile, and decreased time spent swimming in the forced swim test, and decreased ERβ immunoreactivity in the brain than did rats administered ERα AS-ODNs, vehicle, or scrambled AS-ODNs. Rats that were administered ERα AS-ODNs, alone, or with ERβ AS-ODNs had significantly decreased lordosis and decreased ERα immunoreactivity in the brain compared to rats administered ERβ AS-ODNs, vehicle, or scrambled AS-ODNs. Thus, ERβ and ERα may be required for E_2's modulation of affective and sexual behavior, respectively.
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INTRODUCTION

Estrogen's effects in the central nervous system include modulation of affective and sexual behavior. Naturally, sexually receptive rats in proestrus have higher estradiol (E_2) levels and reduced anxiety/depression behaviors compared to diestrous rats (Frye and Bayon, 1999; Frye *et al*, 2000; Frye and Walf, 2002). These effects are mimicked in ovariectomized (ovx) rats administered E_2 regimen that produces proestrous-like levels of E_2 (Pfaff, 2005; Walf and Frye, 2006a). Intracellular E_2 receptors (ERs), the originally identified E_2 binding site (ie ERα) and another ER isoform (ie ERβ; Kuiper *et al*, 1996), are one of E_2's potential substrates for their functional effects. Although DNA and ligand-binding domains are similar, these ER isoforms have distinct encoding genes, effects on gene regulation and patterns of expression (Kuiper *et al*, 1998). As described below, differential distribution of ER isoforms in the brain suggests that E_2 may have ERα- and/or ERβ-specific functional effects.

The hippocampus may be important for E_2's effects on anxiety and depression behaviors. Administration of ER antagonists, which are not specific for ER isoforms, subcutaneously (tamoxifen) or to the hippocampus (ICI 182780) similarly attenuate E_2's anti-anxiety and anti-depressant-like effects (Walf and Frye, 2005b, 2006a). The hippocampus primarily expresses ERβ (Shughrue *et al*, 1997; Shughrue and Merchenthaler, 2001). Studies in ERβ knockout mice suggest that ERβ is required for affective behavior as these mice have increased anxiety and depression behavior, which is not reversed by E_2 administration (Krezel *et al*, 2001; Imwalle *et al*, 2005; Rocha *et al*, 2005; Walf and Frye, 2006a). Administration of selective ER modulators (SERMs) that have greater affinity for ERβ than ERα to ovx rats decrease anxiety and depressive behavior

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when administered subcutaneously or to the hippocampus (Walf *et al*, 2004; Walf and Frye, 2005b, 2007). Thus, ERβ in the hippocampus is a likely target for E_2's actions and effect.

Sexual behavior of female rats may involve actions of ERα in the ventral medial hypothalamus (VMH). The mating posture of female rodents, lordosis, depends upon initiation by E_2 in the VMH (Pfaff, 1980, 1999, 2005). Administration of E_2 to the VMH promotes lordosis and E_2's effects can be obviated by subcutaneous administration of ER antagonists or blockade of ER binding in the VMH (Etgen and Shamamian, 1986; Etgen, 1987; Pleim *et al*, 1989). ER expression in the VMH is primarily ERα (Shughrue *et al*, 1997, 1998). Studies in ER knockout mice and using ERα-specific SERMs suggest that ERα is critical for lordosis (Ogawa *et al*, 1998, 2003; Walf and Frye, 2005b). Thus, ERα in the VMH may be required for the effects of E_2 for lordosis.

Studies using ER knockout mice address the effects of lifetime ER knockdown, but not acute effects in specific brain regions. In the present study, E_2-primed ovx rats were infused ERα and/or ERβ antisense oligodeoxynucleotides (AS-ODNs) intracerebroventricularly (ICV) and tested in the open-field, elevated plus maze, forced swim test, and for sexual receptivity, and brains were collected for immunohistochemical evaluation of hippocampal ERβ expression and hypothalamic ERα expression to verify efficacy of ICV AS-ODN administration. If ERβ in the hippocampus is required for effect, and ERα in the hypothalamus is required for sexual receptivity, then ERα and/or ERβ AS-ODNs should attenuate E_2's effects and reduce immunostaining for ERβ in the hippocampus and ERα in the VMH, respectively.

MATERIALS AND METHODS

These methods were pre-approved by the Institutional Animal Care and Use Committee at SUNY Albany.

Animals and Housing

Adult (55 + days old), female Long-Evans rats ($n = 50$) were obtained from the breeding colony in the Social Sciences and Life Sciences Research Buildings in the Laboratory Animal Care Facility at SUNY-Albany (original stock from Taconic Farms, Germantown, NY). Rats were group-housed (4–5 per cage) in polycarbonate cages ($45 \times 24 \times 21$ cm) in a temperature-controlled room ($21 \pm 1^{\circ}$C). Rats were maintained on a 12/12 h reversed light cycle (lights off at 0800 h) with continuous access to Purina Rat Chow and tap water.

Surgery

Young adult (55 days old) rats were ovx under Rompun (12 mg/kg; Bayer Corp., Shawnee Mission, KS) and Ketaset (80 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) anesthesia. One week later, rats had stereotaxic surgery and were implanted with 23-gauge cannula to the right lateral ventricle (ICV; from bregma AP = 1.0, ML = 1.6, DV = 5.5; as per Paxinos and Watson (1986); Frye and Duncan (1996) under the same anesthesia regimen. Placement of cannula was determined the following day (see below for details) and then rats were E_2-primed, administered AS-ODNs, and behaviorally tested 1 week later.

Determination of Cannula Placement

To determine cannula placement, rats were infused with Angiotensin II (Sigma; 100 ng/μl), which reliably induces drinking behavior when administered to the lateral ventricle (Phillips, 1978). Rats were then tested for their latency to drink and number of licks made during a 3 min test (Anxiometer; Columbus Instruments, Columbus, OH). Rats that did not drink water when infused with Angiotensin II ($n = 3$) were administered saline vehicle in the experiment and their data did not differ from rats that drank water when administered Angiotensin II and were administered saline vehicle in the experiment.

E_2-Priming

All rats were administered 10 μg 17β-E_2 (Steraloids, Newport, RI) dissolved in vegetable oil vehicle 48 h before testing (Walf and Frye, 2005a). This E_2 dosing regimen was chosen as it has previously been shown to increase anti-anxiety, antidepressant-like, and sexual behavior of ovx rats (Walf *et al*, 2004; Walf and Frye, 2005a). Note that although progesterone administration would produce greater sexual responses in E_2-primed ovx rats than E_2 alone, progestins alter the end points utilized and these studies were designed to investigate specifically the effects of E_2.

Experimental Groups

Rats were randomly assigned to one of five experimental groups ($n = 10$/group) that received saline vehicle, scrambled control oligodeoxynucleotides, ERα AS-ODNs, ERβ AS-ODNs, or both ERα and ERβ AS-ODNs.

Infusions

Rats were infused with 1 μl of modified full-length phosphorothioate HPLC-purified mRNA AS-ODNs, scrambled control oligodeoxynucleotides, or saline vehicle three times. Although these AS-ODNs were modified, and should be metabolized less quickly than unmodified ODNs, rats received three infusions throughout E_2-priming. The first infusion was done immediately before administration of E_2 (ie 48 h before behavioral testing), 24 h later, and 30 min before testing (2000 ng/μl in saline; Liang *et al*, 2002). AS-ODNs were obtained from Genomechanix (Gainesville, FL). Sequences were 5′-CATGGTCATGGTCAG-3′ for ERα AS-ODNs, 5′-GAATGTCATAGCTGA-3′ for ERβ AS-ODNs, 5′-ATCGTGGATCGTGAC-3′ for ERα scrambled control AS-ODNs (Liang *et al*, 2002; Walf *et al*, 2006a; Edinger and Frye, 2007).

Behavioral Testing

Data from rats were collected by hand by an observer and/or with a video-tracking system (Any-Maze, Stoelting, Wood Dale, IL). There was a greater than 95% concordance in data collected with these methods and in the two buildings. Rats were tested on one occasion in a battery of all of the following tasks in the order indicated below. Rats were tested between 1200 and 1600 h.

Open Field

The open field ($76 \times 57 \times 35$ cm) has a 48-square grid floor and was situated in a brightly lit room. As per previously published methods, the number of central and peripheral squares, which were summed for total, that each rat entered during the 5 min test were recorded (Frye *et al*, 2000). Total entries made are an index of general motor behavior, and an increased number of central entries is an index of anti-anxiety behavior.

Elevated Plus Maze

The elevated plus maze was situated in a brightly lit room and consisted of four arms (two open without walls and two enclosed by 30 cm high walls) 49 cm long and 10 cm wide, elevated 50 cm off the ground. Rats were placed at the junction of the open and closed arms and the number of entries and time spent on the open and closed arms were recorded (according to Frye *et al*, 2000). Total entries made in the plus maze are an index of general motor behavior and an increase in time spent, and entries made, on the open arms indicates anti-anxiety behavior.

Lordosis Testing

Rats were tested for sexual behavior (ie lordosis quotients) in a Plexiglas chamber ($50 \times 25 \times 30$ cm) in the brightly lit testing room with a sexually experienced adult male. Females were placed in the chamber and the number of lordosis postures assumed when mounted by the male within 10 total mounts or test minutes, whichever came first. The number of times that the female assumed the lordosis posture as a function of the number of mounts made by the male (lordosis quotients) were used as an index of sexual receptivity (Hardy and DeBold, 1971; Walf and Frye, 2005b).

Forced Swim Test

The forced swim test was utilized as per previously published methods (Frye and Walf, 2002). Rats were tested in the forced swim test in a chamber filled with 30 cm of 30°C water. Time spent by the rat struggling to get out of the chamber, swimming underneath the surface of the water, or completely immobile except for the minimal movements necessary to keep nose above water and the number of fecal boli produced by rats were recorded during the 10-min task. Struggling and swimming can be considered as indices of general motor behavior and a decrease in time spent immobile indicates anti-depressant-like behavior. The number of fecal boli is considered an ethologically relevant index of anxiety/fear in rodents.

Perfusion

Immediately following testing, rats were overdosed on sodium pentobarbital (150 mg/kg; perfused via intra-cardiac puncture with a needle attached to tubing that pumped 100 ml phosphate-buffered saline (PBS) and then 250 ml of fixative (4% paraformaldehyde) through tissue. Brains were stored in fixative and then in a 30% sucrose PBS solution followed by rinsing and storage in 0.2 M PBS.

Immunohistochemistry

The behavioral data obtained suggest that concentrations of AS-ODNs (which can sharply decline away from the ventricle after infusion) utilized were effective. Immunohistochemical analyses of brain regions that are known to be involved in E_2's effects on anxiety and reproduction, the hippocampus (Walf and Frye, 2007) and VMH (Pleim *et al*, 1989), were performed. Brains were cut on coronal sections at 50 μm using a Vibratome. After obtaining all the sections, two separate immunohistochemistry assays for ERα and ERβ, respectively, were performed as per modified methods (Garcia-Ovejero *et al*, 2002). The primary antisera used to detect the antigen was ERα, MC20, purchased from Santa Cruz Biotechnology (Santa Cruz, CA), diluted 1 μg/ml; ERβ, purchased from Affinity Bioreagents, diluted 1 : 2000. Rinses and incubations were done on free-floating sections under moderate shaking. Sections were first rinsed in 0.1 M phosphate-buffered saline (pH 7.4), containing 0.3% Triton X-100 and 0.3% Bovine albumin serum; subsequent washes used this same rinsing solution. Endogenous peroxidase activity was inhibited by incubating the sections in 0.1 M phosphate buffer containing 0.9% H_2O_2 and 30% methanol. After shaking in the rinsing solutions three times for 10 min each wash, sections from both immunohistochemistry assays were incubated separately for two nights in the rabbit IgG polyclonal primary antisera against ERα and ERβ in 3% normal goat serum. After careful washes, sections were incubated for 2 h at room temperature with biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA; 1:250). Sections were incubated for 90 min with a complex avidine-biotin peroxidase, diluted 1:300 (ABC, Pierce). Peroxidase reaction product was revealed by incubating the sections in a solution of 0.03% diaminobenzidine and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. Sections were dehydrated and mounted carefully and subsequently examined on a Leica DMRB-E microscope.

Morphometric Analysis

The number of ERα and ERβ immunoreactive neurons in CA1 pyramidal layer of the hippocampus and in the ventromedial nucleus of the hypothalamus was estimated by the optical disector method (Howard and Reed, 1998) using total section thickness for disector height (Hatton and von Bartheld, 1999) and a counting frame of 55×55 μm. A total of 10 counting frames were assessed per animal. Section thickness was measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221; Traunreut, Germany) attached to the stage of a Leitz microscope. Cell nuclei from immunoreactive neurons that came into focus while focusing down through the disector height were counted. All counts were performed blind.

Statistical Analyses

One-way analyses of variance (ANOVAs) were used to examine effects of ICV treatment for behavioral end points

and number of immunoreactive cells in the brain. To take into account differences in general motor activity in the tasks utilized, measures of affective behavior in the open field (central entries), elevated plus maze (open arm entries), and forced swim test (immobility), were standardized (expressed as % of total activity) and are depicted in Figures 1–3. Raw means (±SD; SEM) of these measures and general motor measures are included in the results section and Table 1, respectively. Given that multiple comparisons were made, Tukey's *post hoc* tests were utilized to determine group differences, as appropriate. Differences were considered significant when $P<0.05$.

RESULTS

Open Field

There was a main effect of infusion condition for central entries made, as a function of total entries made, in the open field ($F(4,45)=23.73$, $P<0.01$) (see Figure 1). *Post hoc* tests revealed that infusions of ERβ (5.9±4.5 SD; 1.4 SEM) or ERα/ERβ (4.8±6.0 SD; 1.9 SEM) AS-ODNs significantly decreased central entries compared to infusions of ERα AS-ODNs (33.9±15.4 SD; 4.9 SEM), saline vehicle (28.5±12.9 SD; 4.1 SEM), or scrambled control AS-ODNs (27.6±12.8 SD; 4.0 SEM).

There was a main effect of infusion condition to alter total entries made in the open field, such that rats administered ERα AS-ODNs made more total entries than did rats administered ERα/ERβ AS-ODNs infusions ($F(4,45)=3.63$, $P<0.01$) (Table 1).

Elevated Plus Maze

There was a main effect of AS-ODN infusions to alter the number of open arm entries, expressed as a percentage of total arm entries in the plus maze ($F(4,45)=6.04$, $P<0.01$) (Figure 2). *Post hoc* test revealed that infusions of ERβ (1.7±1.6 SD; 0.5 SEM) or ERα/ERβ (1.1±1.2 SD; 0.4 SEM) AS-ODNs significantly decreased the entries made on the open arms of the elevated plus maze compared to infusions of ERα AS-ODNs (3.5±2.0 SD; 0.6 SEM), saline vehicle (2.8±1.2 SD; 0.4 SEM), or scrambled control AS-ODNs (2.8±1.3 SD; 0.4 SEM). A similar pattern was observed for open arm time ($F(4,45)=9.03$, $P<0.01$), such that infusions of ERβ (15.7±17.5 SD; 5.5 SEM) or ERα/ERβ (4.6±6.5 SD; 2.0 SEM) AS-ODNs significantly decreased open arms time compared to that seen in rats infused with ERα AS-ODNs (46.7±28.3 SD; 8.9 SEM), saline vehicle (52.3±27.6 SD; 8.7 SEM), or scrambled control AS-ODNs (45.8±25.0 SD; 7.9 SEM).

ICV infusions of ERα AS-ODNs significantly increased total, but not closed, arm entries made in the elevated plus maze compared to infusions of ERα/ERβ AS-ODN ($F(4,45)=2.73$, $P<0.04$) (Table 1).

Forced Swim Test

There was a main effect of infusion condition for duration of immobility (as a function of time spent swimming) in the forced swim test ($F(4,45)=9.21$. $P<0.01$) (Figure 3). Infusions of ERβ (328.2±33.3 SD; 10.5 SEM) and ERα/ERβ (325.9±48.96 SD; 15.48 SEM) AS-ODNs significantly

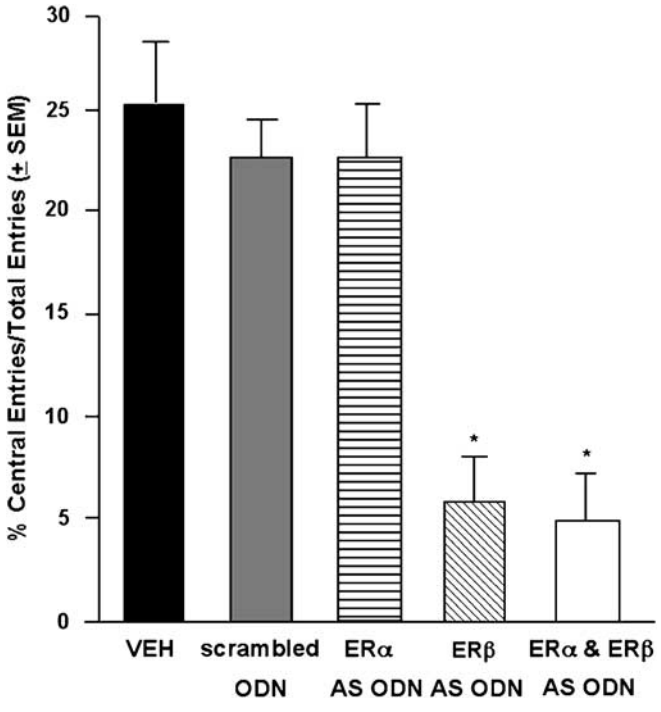

Figure 1 The mean (±SEM) central entries/total entries made in a brightly lit open field. *Above bar indicates a significant difference ($P<0.05$) from rats administered ERβ AS-0DNs (5.2 SD) and ERα/ERβ AS-ODNS (4.3 SD) *vs* vehicle (8.5 SD), scrambled control (7.1 SD) and ERα AS-ODNS (8.6 SD).

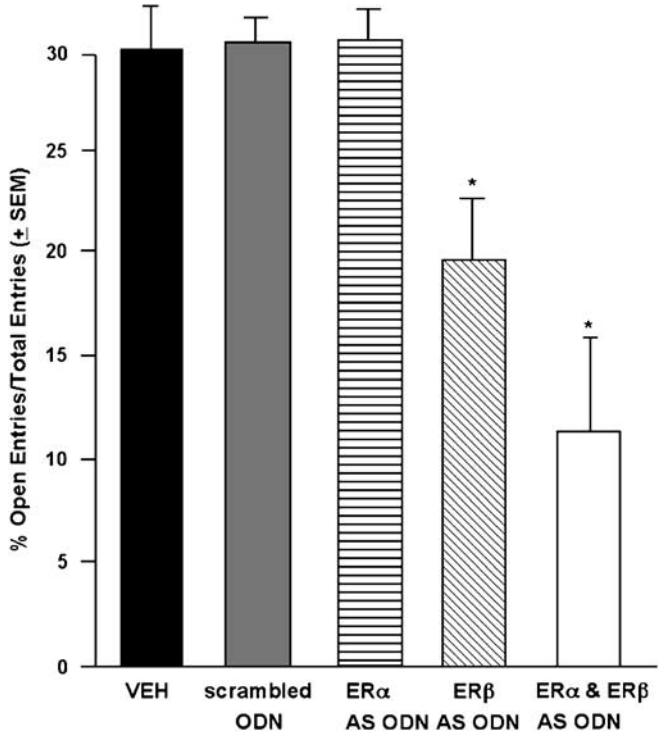

Figure 2 The mean (±SEM) open arm entries made in the elevated plus maze. *Above bar indicates a significant difference ($P<0.05$) from rats administered ERβ AS-0DNs (17.1 SD) and ERα/ERβ AS-ODNS (12.5 SD) *vs* vehicle (7.7 SD), scrambled control (6.0 SD) and ERα AS-ODNS (11.2 SD).

increased the duration spent immobile compared to that observed in rats administered infusions of saline vehicle (190.5 ± 21.99 SD; 6.9 SEM), scrambled control AS-ODNs (180.3 ± 38.7 SD; 12.2 SEM), or ERα AS-ODNs (181.4 ± 22.9 SD; 7.3 SEM).

ICV infusions of ERα/ERβ AS-ODN significantly decreased duration spent swimming compared to infusions of saline vehicle, scrambled AS-ODNS, or ERα AS-ODNs ($F(4,45) = 11.7$, $P < 0.01$) (Table 1). There were no significant differences between groups for duration spent struggling in the forced swim test (Table 1).

Although rats administered ERβ AS-ODNS ICV had the greatest mean number of fecal boli produced in the forced swim test, statistical analyses of group differences for this measure did not reach statistical significance ($P = 0.06$; data not shown).

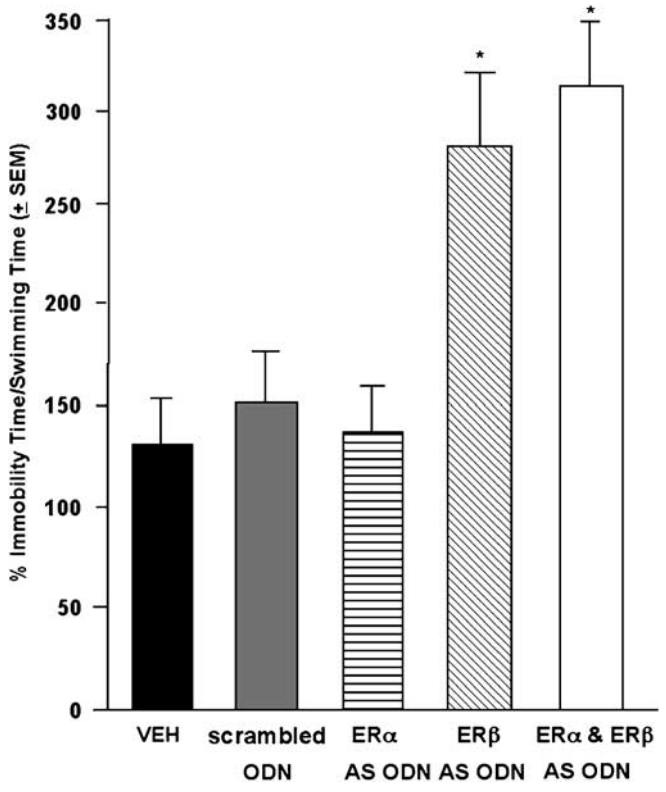

Figure 3 The mean (±SEM) time spent immobile in the forced swim test. *Above bar indicates a significant difference ($P < 0.05$) from rats administered ERβ AS-0DNs (90.0 SD) and ERα/ERβ AS-ODNS (145.6 SD) *vs* vehicle (50.8 SD), scrambled control (69.1 SD) and ERα AS-ODNS (49.9 SD).

ERβ Immunoreactivity

Effectiveness of AS-ODNs utilized was verified by reduced ERβ immunoreactivity in VMH ($F(3,8) = 323.7$, $P < 0.01$) and hippocampal ($F(3,8) = 36.8$, $P < 0.01$) (Figure 4) sections of rats administered ICV ERβ or ERα/ERβ AS-ODNs compared to that in rats administered ICV saline or scrambled AS-ODNs (Table 2).

Sexual Receptivity Test

There was a main effect of infusion condition for lordosis quotients, such that infusions of ERα and ERα/ERβ AS-ODNs significantly decreased lordosis quotients compared to infusions of saline vehicle, scrambled control AS-ODNs, or ERβ AS-ODNs ($F(4,45) = 9.31$, $P < 0.01$) (Figure 5).

ERα Immunoreactivity

Effectiveness of AS-ODNs utilized was verified by lowered ERα immunoreactivity in the VMH ($F(3,8) = 238.5$, $P < 0.01$) (Figure 6) and hippocampus ($F(3,8) = 46.2$, $P < 0.01$) of rats administered ICV ERα or ERα/ERβ AS-ODNs compared to those administered ICV saline or scrambled AS-ODNs (Table 2).

DISCUSSION

These results supported our hypothesis that E_2's effects for anxiety/depressive and sexual behavior may be through actions involving ERβ and ERα, respectively. In support, rats administered ERβ or ERα/ERβ AS-ODNs ICV had decreased central entries in the open field, decreased open arm time/entries in the plus maze, increased time spent immobile in the forced swim test, and decreased immuno-

Table 1 Indices of Motor Behavior (mean ± SD; SEM): Total Square Entries Made in the Open Field, Total Arm Entries Made in the Elevated Plus Maze, and Time Spent Struggling in the Forced Swim Test of Rats ICV Administered Vehicle or Scrambled, ERα, ERβ, or ERα/ERβ Antisense Oligodeoxynucleotides (AS-ODNs)

	Open field	Elevated plus maze	Forced swim test	
Condition	**Total entries**	**Total entries**	**Time swimming (s)**	**Time struggling (s)**
Vehicle	109 ± 25;8	9 ± 2;1	244 ± 86;27	166 ± 80;26
Scrambled AS-ODNs	125 ± 53;17	8 ± 3;1	277 ± 47;15	137 ± 41;13
ERα AS-ODNS	148 ± 37;12*	10 ± 3;1*	272 ± 42;13	146 ± 38;12
ERβ AS-ODNs	119 ± 119;7	7 ± 3;1	142 ± 64;20@	130 ± 46;15
ERα/ERβ AS-ODNs	85 ± 44;14	6 ± 3;1	153 ± 53;17@	121 ± 41;13

$n = 10$/group.
*$P < 0.05$ compared to infusions of ERα/ERβ AS-ODNs.
@$P < 0.05$ compared to infusions of saline, scrambled, or ERα AS-ODNs.

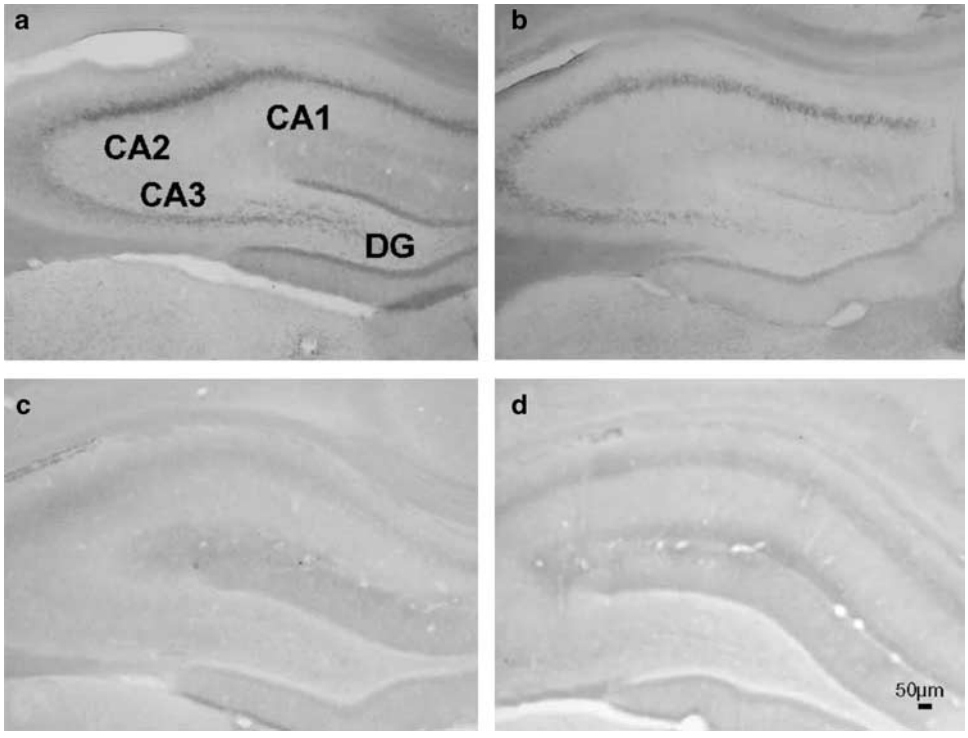

Figure 4 ERβ immunoreactivity is greater in the hippocampus of rats administered saline vehicle (a), scrambled control AS-ODNs (b) *vs* ERβ AS-ODNs (c), or ERα and ERβ AS-ODNs (d). Scale bar is 50 μm.

Table 2 The Number of ERα or ERβ Immunoreactive Cells/mm^3 in the Ventral Medial Hypothalamus (VMH) or CA1 Region of the Hippocampus (mean ± SD; SEM) of Rats ICV Administered Scrambled, ERα, ERβ, or ERα/ERβ Antisense Oligodeoxynucleotides (AS-ODNs)

	ERα-immunoractive cells		ERβ-immunoreactive cells	
Condition	**VMH**	**CA1**	**VMH**	**CA1**
Scrambled AS-ODNs	48042 ± 4704;2716	32866 ± 4176;2411	11869 ± 1154;666	28846 ± 3079;1777
ERα AS-ODNS	9598 ± 1185;684*	7607 ± 885;511*	13170 ± 671;387	30312 ± 8831;5098
ERβ AS-ODNs	51070 ± 1824;1053	35192 ± 6409;3700	379 ± 83;47$^{@}$	1307 ± 594;343$^{@}$
ERα/ERβ AS-ODNs	8736 ± 757;437*	8011 ± 631;364*	373 ± 202;117$^{@}$	1067 ± 110;64$^{@}$

$n = 3$/group.
*$P < 0.05$ compared to infusions of scrambled or ERβ AS-ODNs.
$^{@}P < 0.05$ compared to infusions of scrambled or ERα AS-ODNs.

reactivity for ERβ in the brain (VMH and CA1) compared to rats administered saline vehicle or scrambled control AS-ODNs, or ERα AS-ODNs. These effects were not solely due to gross changes in motor behavior of rats in these tasks. Rats administered ERα or ERα/ERβ AS-ODNs ICV had decreased lordosis quotients and immunoreactivity for ERα in the VMH and hippocampus compared to rats administered saline vehicle or scrambled control AS-ODNs, or ERβ AS-ODNs. Together, these results suggest the importance of ERβ for affective behavior and ERα for sexual receptivity.

The present study using a short-term regimen of AS-ODNs targeted towards ERs contributes to the existing literature on the role of ERα and/or ERβ knockdown for behavior. Studies utilizing transgenic mice that do not express ERα and/or ERβ demonstrate that knockout of these receptors throughout the lifespan have specific behavioral effects. ERβ, but not ERα, knockout mice have increased anxiety and depressive behavior compared to their wild type counterparts (Krezel *et al*, 2001; Imwalle *et al*, 2005; Rocha *et al*, 2005; Walf and Frye, 2006a), but their reproductive behavior is intact (Ogawa *et al*, 1999). On the other hand, ERα knockout mice are infertile and are not sexually receptive when in contact with a sexually experienced male (Ogawa *et al*, 1998; Ogawa *et al*, 2003). Indeed, a similar pattern of behavioral responses was observed in ovx, E_2-primed rats in the present study that were administered ERα AS-ODNs. Administration of ERβ AS-ODNs three times throughout the 48 h of E_2-priming before behavioral testing attenuated the anti-anxiety and antidepressive effects of E_2, but did not alter lordosis.

Whereas, ERα AS-ODNs administered for the same time period attenuated lordosis quotients without altering anxiety and depressive responses. Thus, these data suggest that there are specific functional effects of E_2 at ERα and ERβ, but the possibility ERα and ERβ that share some cross-regulatory actions for E_2's behavioral effects remains to be addressed.

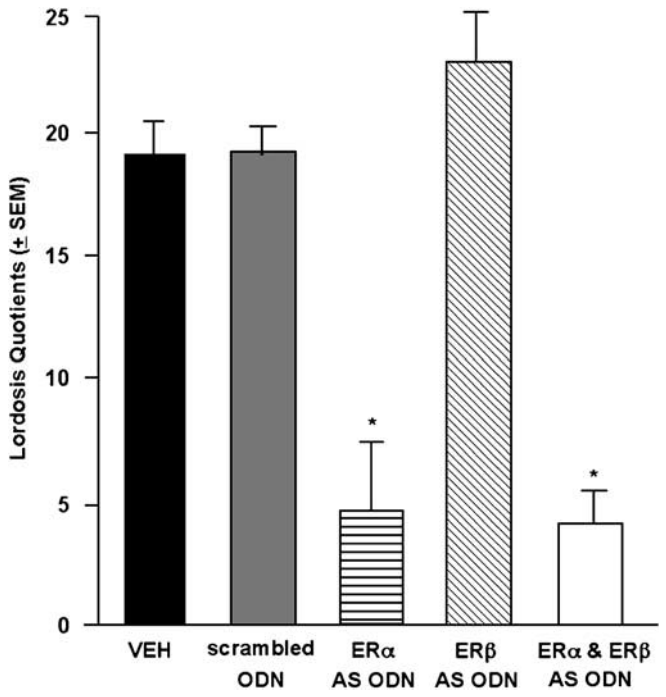

Figure 5 The mean (±SEM) lordosis quotients of rats. *Above bar indicates a significant difference ($P<0.05$) from rats administered ERα AS-0DNs (9.6 SD) and ERα/ERβ AS-ODNS (7.8 SD) *vs* vehicle (10.3 SD), scrambled control (10.2 SD) and ERβ AS-ODNS (6.8 SD).

The results of this study confirm previous studies from our laboratory and others on the role of ERα and ERβ for specific behavioral processes in adult rodents and extend these findings to suggest some brain targets for these effects, the hippocampus, and VMH; albeit, in the present study, ER expression in these brain areas was investigated as a means to determine effectiveness of AS-ODNs strategy employed to knockdown expression of ERα and ERβ in the brain. In the present study, ovx, E_2-primed rats administered ERβ AS-ODNs had decreased anti-anxiety and anti-depressive behavior and reduced ERβ-immunoreactivity in the brain, compared to rats administered control infusions or ERα AS-ODNs demonstrating that ERβ-ODNs were effective in knocking down ERβ. Previous work has shown that systemic administration of 17β-E_2, which would be expected to affect the whole brain, produces similar effects to increase anti-anxiety and anti-depressant-like behavior of ovx rodents as does direct hippocampal administration of E_2 (Slater and Blizard, 1976; Rachman *et al*, 1998; Frye and Wawrzycki, 2003; Frye and Walf, 2004; Walf *et al*, 2004; Walf and Frye, 2005a, b, 2006a, 2007). Effectiveness of ERα AS-ODN administration to knock down ERα is revealed by reduced ERα immunoreactive cells in the brain in ovx, E_2-primed rats administered ERα AS-ODNs compared to rats administered control infusions or ERβAS-ODNs demonstrating that ERα-ODNs were effective in knocking down ERα. Indeed, E_2 administration to the VMH increases lordosis of ovx female rodents (Pleim *et al*, 1989). Given that 17β-E_2 has equal affinity for ERα and ERβ, an interesting question is whether the observed effects for 17β-E_2 when administered to the hippocampus or VMH are also observed with ERα or ERβ specific SERMS. We have recently shown that SERMs that are selective for ERβ decrease anxiety and depressive behavior when administered to the hippocampus, but not to a control missed site that expresses ERβ, the ventral tegmental area; these effects

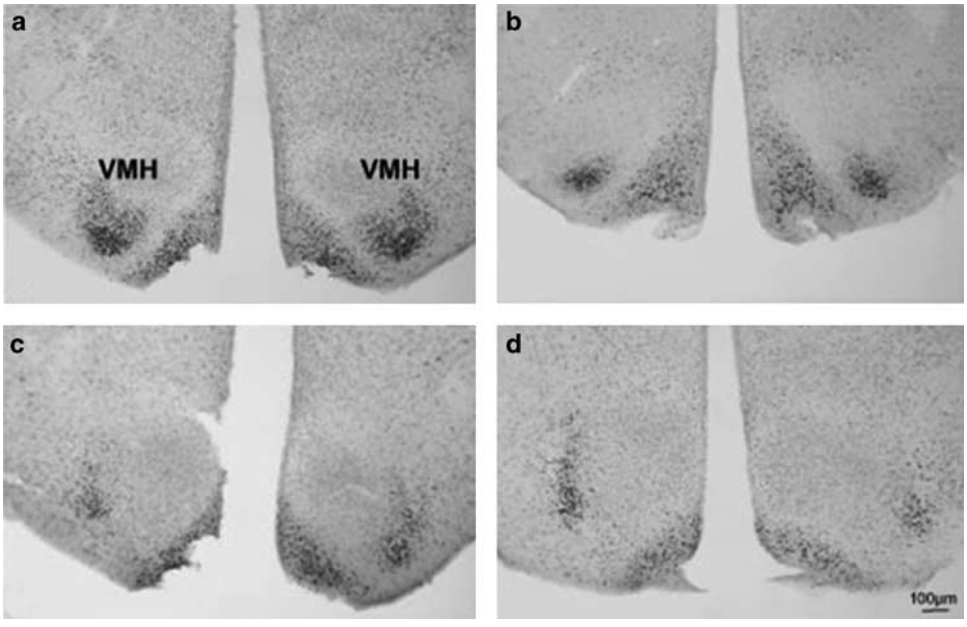

Figure 6 ERα immunoreactivity is greater in the hypothalamus of rats administered saline vehicle (a), scrambled control AS-ODNs (b) *vs* ERα AS-ODNs (c), or ERα and ERβ AS-ODNs (d). Scale bar is 100 μm.

are similar to that observed with subcutaneous administration of SERMs and ERα-specific SERMs were ineffective (Lund *et al*, 2005; Walf *et al*, 2004; Walf and Frye, 2005b, 2007). Furthermore, subcutaneous administration of ERα-, but not ERβ-, SERMs increase lordosis similar to that observed in ovx rats administered 17β-E_2 (Walf and Frye, 2005b). Together, these data suggest that ERβ in the hippocampus and ERα in the VMH may be important sites to investigate more directly for E_2's modulation of affective and socio-sexual behavior, respectively.

These data suggest that actions of ERβ and ERα are necessary and sufficient for E_2's effects on anxiety/depression and sexual behavior, respectively; however, a few issues regarding this interpretation need to be addressed. First, other factors that may have influenced the results observed were not investigated in the present study. As one example, oxytocin may be involved in the effects observed. E_2 induces the oxytocin gene, which is dependent upon ERβ, and oxytocin is involved in both affective and social responses (Nomura *et al*, 2002; Choleris *et al*, 2003). Furthermore, examining the effects of ER AS-ODN treatment to vehicle-administered ovx rats would provide additional information about the importance of ER function for these behavioral effects. Indeed, the downstream effectors of ERs, which may be membrane-bound and/or have membrane actions that potentiate intracellular events, such as extracellular regulated kinase/mitogen-activated protein kinase, need to be elucidated (Wade and Dorsa, 2003; Bryant *et al*, 2005; Vasudevan *et al*, 2001, 2005; Mhyre and Dorsa, 2006). In the future, it would be important to address these points. Second, although the hippocampus and VMH are logical areas to begin investigating the brain region- and ER subtype-specific effects of E_2 for anxiety, and socio-sexual behavior, it is likely that these are not the only brain regions involved in the behavioral effects observed. For instance, direct E_2 administration to the medial amygdala or median raphe nucleus decreases anxiety and depressive behavior of ovx rats demonstrating that these brain regions are sensitive to E_2's effects and involved in affective responses (Walf and Frye, 2003; Andrade *et al*, 2005; Walf and Frye, 2006). Indeed, in the case of the amygdala, ERα and ERβ expression varies with reproductive status and mating stimuli (Greco *et al*, 2003a, b). In the present study, immunohistochemistry was utilized as a means to verify that ICV administration of ER AS ODNs produced qualitative changes in expression of ERα and ERβ in the brain. Although Western blot analyses, which would provide another biochemical indicator of the effectiveness of AS-ODN treatment, could not be performed on subjects from the present study, we have previously demonstrated with Western blotting that administration of these ER AS ODNs directly to the striatum or hippocampus reduce ER levels in these regions concomitant with robust behavioral changes (Walf *et al*, 2006a; Edinger and Frye, 2007). Future studies could further investigate whether such treatments, when infused ICV or directly to these brain regions of interest, produce qualitative and quantitative changes in receptor expression using both Western blotting and immunohistochemistry. Third, the known effects for activity/arousal of E_2s, which are clearly dependent upon the environmental context before and during behavioral assessment (see Morgan *et al*, 2004 for a review), may have influenced the present results. High circulating E_2 levels, as occurred during behavioral estrous or when E_2 is administered to ovx rodents, increase spontaneous motor activity (Joyce and Van Hartesveldt, 1984; Becker, 1990; Becker *et al*, 1987; Morgan and Pfaff, 2001, 2002), and these effects may be ERα-specific (Ogawa *et al*, 2003). However, a different pattern of effects for motor activity is observed in a novel open field and/or in different lighting (see Morgan *et al*, 2004 for a review), and these effects in tasks involving novelty/anxiety, such as the open field, may be primarily mediated by E_2's actions at ERβ (Krezel *et al*, 2001). In the present study, when motor behavior in the tasks was compared between groups, a distinct pattern to account for changes in the anxiety or depression measures utilized was not found across all tasks. For instance, infusions of ERα AS-ODNs increased total entries in the open field and elevated plus maze compared to infusions of ERα/ERβ AS-ODNs, but behavior of other groups was similar. In the forced swim test, infusions of ERβ or ERα/ERβ AS-ODNs decreased swimming compared to all other groups, but there was no effect on struggling behavior in the task. Additionally, it is important to note that there was no evidence for non-specific behavioral effects of the AS-ODNs, which may have obviated interpretation of the behavioral effects observed. Indeed, further investigation of ER mechanisms, brain regions of interest, role of context, and effects of arousal/activity for behavioral effects observed is warranted.

These data are interesting as they begin to dissociate mechanisms of E_2 at ERα and ERβ as well as brain targets for these effects. The lifetime prevalence rates for anxiety and depression disorders among women are approximately twice than that seen in men (Breslau *et al*, 1995; Earls, 1987; Kessler *et al*, 1993, 1994; Nolen-Hoeksema, 1987; Schneier *et al*, 1992; reviewed in Seeman, 1997; Young, 1998; Young and Korszun, 2002). Studies in humans suggest that differences in ER isoform expression in the brain may have some functional importance for affective disorders and efficacy in their treatment (Osterlund and Hurd, 2001; Osterlund *et al*, 2000, 2005). E_2 administration to women with anxiety or depressive disorders can enhance mood (reviewed in Walf and Frye, 2006). However, a serious criticism of E_2-based therapies are their potential to enhance proliferation in E_2-sensitive reproductive tissues, which are mediated primarily via actions at ERα (Gustafsson, 2003). Indeed, these findings underscore the importance of further investigating the tissue-specific functional effects of ERα and ERβ.

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