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## Motivated behaviors and levels of 3 $\alpha$ ,5 $\alpha$ -THP in the midbrain are attenuated by knocking down expression of pregnane xenobiotic receptor in the midbrain ventral tegmental area of proestrous rats

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### Abstract

**Introduction**—Progesterone (P<sub>4</sub>) and its product, 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (3 $\alpha$ ,5 $\alpha$ -THP), act in the midbrain ventral tegmental area (VTA) to alter motivated behaviors, such as mating, and motor and anxiety behavior. Of interest is whether 3 $\alpha$ ,5 $\alpha$ -THP formation requires the pregnane xenobiotic receptor (PXR), which is expressed in the midbrain of rats.

**Aim**—The role of PXR in the midbrain for 3 $\alpha$ ,5 $\alpha$ -THP formation, which precedes modulation of motivated behaviors, was investigated.

**Methods**—Rats had estrous cycle phase determined and were assessed when they were in diestrus or proestrus. Diestrus and proestrous rats were infused with control or anti-sense oligodeoxyribonucleotides (AS-ODNs) targeted against PXR to the VTA.

**Main Outcome Measures**—In pilot studies, PXR gene and protein expression in the midbrain was determined with quantitative RT-PCR and western blotting, respectively. Diestrus and proestrous rats infused with control or AS-ODNs to the VTA were tested for anxiety (open field, plus maze), social (social interaction) and sexual (paced mating) behavior. Expression of PXR in the midbrain was verified with Western blotting. Plasma estradiol, P<sub>4</sub>, dihydroprogesterone (DHP), and 3 $\alpha$ ,5 $\alpha$ -THP levels, and brain P<sub>4</sub>, DHP, and 3 $\alpha$ ,5 $\alpha$ -THP levels, were measured. We predicted that proestrous rats infused with PXR AS-ODNs would have decreased anti-anxiety, social, and sexual behavior, lower midbrain expression of PXR, and lower midbrain levels of 3 $\alpha$ ,5 $\alpha$ -THP, compared to controls.

**Results**—Results supported the hypothesis that formation of 3 $\alpha$ ,5 $\alpha$ -THP requires PXR, and may be important for motivated behaviors. PXR AS-ODN, compared to control, infusions to the VTA reduced PXR expression and 3 $\alpha$ ,5 $\alpha$ -THP levels in the midbrain, and attenuated sexual receptivity of proestrous rats.

**Conclusions**—Knockdown of PXR in the midbrain reduces 3 $\alpha$ ,5 $\alpha$ -THP levels and sexual receptivity of proestrous rats. Thus, PXR in the midbrain may be required for the observed

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increase in 3 $\alpha$ -5 $\alpha$ -THP during proestrus, which has subsequent effects on motivated, reproductive behaviors.

## Keywords

Allopregnanolone; Lordosis; Progesterone; Neurosteroids; Mating; Receptivity

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## Introduction

A focus on the functional role of progesterone (P<sub>4</sub>) has been on its pregnancy-maintaining effects; however, it is clear that P<sub>4</sub>, and its products, have effects beyond pregnancy for motivated behaviors, encompassing social interaction, mating, and other reproduction-relevant behaviors. One brain target of progestogens, such as P<sub>4</sub> and 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (3 $\alpha$ ,5 $\alpha$ -THP), is the midbrain VTA. Natural elevations of P<sub>4</sub> and 3 $\alpha$ ,5 $\alpha$ -THP across the estrous cycle coincide with increases in motivated behaviors, such as mating and reproduction-relevant behaviors (e.g. social interaction, anxiety), during proestrus [1–7]. Moreover, the effects of exogenous administration of progestogens support the role that they play for motivation. For example, administration of 3 $\alpha$ ,5 $\alpha$ -THP to ovariectomized rats enhances mating, pro-social behavior, and anti-anxiety-like responses [4]. Results from other animal models corroborate these findings that progestogens can alter motivated processes. For instance, 3 $\alpha$ ,5 $\alpha$ -THP produces a place preference [2,8,9] and rats will preferentially self-administer 3 $\alpha$ ,5 $\alpha$ -THP over water [10]. Progestogens have effects to amplify motivational processes, such as those observed with cocaine. In support, proestrous rats have greater responses to cocaine than do diestrous rats [11]. Among ovariectomized rats, P<sub>4</sub> produces sex-dependent effects on behavioral responses to cocaine [12,13] and sequential administration of estradiol (E<sub>2</sub>) and P<sub>4</sub> increases cocaine self-administration and tends to increase behavioral sensitivity to cocaine [14]. Thus, progestogens play a role in motivated behaviors.

Determining the source of 3 $\alpha$ ,5 $\alpha$ -THP is critical to investigating the role and mechanisms of 3 $\alpha$ ,5 $\alpha$ -THP for anxiety, social, and reproductive processes. 3 $\alpha$ ,5 $\alpha$ -THP is formed via activation of a metabolic and/or biosynthetic pathway. For example, in the VTA, ovarian P<sub>4</sub> is readily metabolized to dihydroprogesterone (DHP) by 5 $\alpha$ -reductase and DHP is converted to 3 $\alpha$ ,5 $\alpha$ -THP by 3 $\alpha$ -hydroxysteroid dehydrogenase. Blocking P<sub>4</sub>'s metabolism to 3 $\alpha$ ,5 $\alpha$ -THP pharmacologically, or with knockout mouse models, inhibits progestogens' facilitating effects on reproductive, social, and anti-anxiety behaviors, and 3 $\alpha$ ,5 $\alpha$ -THP-replacement can produce the opposite effects [1,15–18]. For female rats, engaging in mating promotes biosynthesis, or neurosteroidogenesis, of 3 $\alpha$ ,5 $\alpha$ -THP from the precursor, cholesterol in the midbrain VTA, as well as regions important for reward, emotion, and cognition (i.e. striatum, hippocampus, and cortex) [4,16,19]. The 18-kDa translocator protein (TSPO; formerly known as the peripheral-type benzodiazepine receptor) is essential for neurosteroidogenesis in that it transports cholesterol to cytochrome P450-dependent side chain cleavage enzymes (P450<sub>scc</sub>) [20–22]. The steroidogenic acute regulatory (StAR) protein is important for stimulating this action, but the precise mechanisms are unknown. Inhibiting formation of 3 $\alpha$ ,5 $\alpha$ -THP through any of these metabolic pathways in the VTA attenuates these neuroendocrine and behavioral effects [1,16,18,23]. Thus, the effects of 3 $\alpha$ ,5 $\alpha$ -THP on reproductive, social and anxiety behaviors occur following its generation from the metabolism of ovarian P<sub>4</sub>, which infiltrates the VTA, as well as from de novo synthesis from cholesterol within the brain.

It is generally accepted that progestogens are synthesized in the brain and peripheral nerves, but other factors that may regulate steroid biosynthesis are poorly understood. The Pregnane Xenobiotic Receptor (PXR), a promiscuous nuclear receptor, binds 3 $\alpha$ ,5 $\alpha$ -THP and can

mediate transcription of cytochrome (CYP) enzymes [24–26]. CYP enzymes are involved in biosynthesis of steroids. The rodent PXR is analogous to the Steroid and Xenobiotic Receptor (SXR) in humans, also referred to as the human-PXR. Whether behavioral and/or neuroendocrine variations in midbrain 3 $\alpha$ ,5 $\alpha$ -THP occur concomitant with, and/or require, PXR is of interest.

## Aims and Hypotheses

Our hypothesis is that formation of 3 $\alpha$ ,5 $\alpha$ -THP requires PXR in the midbrain. First, we determined that there is expression of PXR gene and protein in the proestrous rat midbrain with quantitative reverse transcriptase polymerase chain reaction (qPCR) and Western blotting, respectively. Second, to determine whether PXR may underlie some of 3 $\alpha$ ,5 $\alpha$ -THP's actions in the midbrain VTA, diestrous and proestrous rats were administered placebo control or PXR AS-ODNs via infusions to the midbrain VTA. Following infusions to the midbrain, motivated and socially-relevant behaviors (open field, elevated plus maze, social interaction, and paced mating) of rats were assessed. For the open field and elevated plus maze, measures of anti-anxiety behavior were determined as a function of motor behavior in these tasks. Social behavior was assessed in the social interaction task and reproductive behavior was assessed in the paced mating task. Among behaviorally-tested rats administered control and PXR AS-ODN infusions, expression of PXR in the midbrain was verified with western blotting, plasma levels of E<sub>2</sub>, P<sub>4</sub>, DHP, and 3 $\alpha$ ,5 $\alpha$ -THP were measured, and brain (cortex, hippocampus, striatum, hypothalamus, and midbrain) levels of P<sub>4</sub>, DHP, and 3 $\alpha$ ,5 $\alpha$ -THP were determined.

## Methods and Main Outcome Measures

These methods utilizing live animals (surgery, drug manipulations, behavioral testing, euthanasia) were approved by the Institutional Animal Care and Use Committee at The University at XXXX and were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

## Experimental Overview

In pilot experiments to assess suitability of PXR AS-ODNs for knocking down expression of PXR in the midbrain, PXR mRNA and protein expression in the midbrain VTA was determined among diestrous and proestrous rats using qPCR (n=2–4/group) and Western blotting (n=5/group). PXR mRNA expression was determined in the midbrain VTA among diestrous and proestrous rats infused with PXR AS-ODN (diestrous n=2, proestrous n=2), compared to vehicle infusions (diestrous n=4, proestrous n=4), using qPCR. Typical western blotting methods were utilized to assess PXR protein expression in the midbrain of proestrous and diestrous rats. As described in the Results, rats administered AS-ODNs to the VTA had lower expression (as indicated as a fold-change from control) than did those administered vehicle; however, there was large variability in these groups. Data investigating PXR protein levels were more robust. These pilot results substantiated assessing PXR protein expression with western blotting in this experiment. As such, behavior, hormone levels, and PXR protein expression of diestrous and proestrous rats administered control or PXR AS-ODN infusions to the VTA are described herein.

## Animal Housing

Adult (55–60 days old), Long-Evans female rats (N=88) were bred in the Life Sciences Laboratory Animal Care Facility at The University at Albany-SUNY (original stock obtained from Taconic, Germantown, NY, USA). Rats were group-housed (n=3–5/cage) in polycarbonate cages with woodchip bedding (45 × 24 × 21 cm) in a temperature-controlled

room ( $21 \pm 1$  °C) and were maintained on a 12:12 h reversed light cycle (lights off at 08:00 am). Rats had continuous access to Purina Rat Chow and tap water in their home cages.

### Estrous Cycle

On each day during the experiment (between 8:00 and 10:00 am), rats were “cycled”, in that vaginal epithelium of each rat was collected and examined under a light microscope for presence of different cell types that predominant in each stage of the estrous cycle. Rats were cycled through two normal estrous cycles (4–5 day cycle) prior to testing. Rats were tested on either proestrus (epithelium characterized by nucleated cells, 4–5 days after the previous occurrence) or diestrus (heterogeneous cell types in vaginal epithelium for two consecutive days, which transpired 4–5 days after the previous 2-days of diestrus).

### Surgical Protocol

Some rats ( $n=78$ , which does not include the 10 cycling rats that were utilized in the pilot western blotting experiment) were stereotaxically implanted, under xylazine (12 mg/kg) and ketamine (80 mg/kg) anesthesia, with bilateral guide cannulae aimed at the VTA (from bregma: AP =  $-5.3$ , ML =  $0.4$ , DV =  $-7.0$ , as per [27]). Guide cannulae consisted of 23-gauge stainless steel needles, with tips sanded smooth, with 30-gauge stainless steel removable inserts. Rats recovered from surgery for an average of 10 days. After surgery, rats were evaluated daily for their ability to right themselves and cage-climb, presence of proper muscle tone, and reflexive responses to hindlimb extension. Rats were also evaluated for weight gain after surgery. Only rats that passed these neurological evaluations and gained weight following surgery until behavioral testing commenced were continued in the experiment.

### Infusion Condition

Proestrous rats were randomly-assigned to be administered 1  $\mu$ l control ( $n=20$ ) or PXR AS-ODN ( $n=13$ ; 5'CTTGCGGAAGGGGCACCTCA3'; 250 ng/1  $\mu$ l) infusions and diestrus rats were randomly-assigned to be infused with control ( $n=17$ ) or PXR AS-ODN ( $n=16$ ) infusions. Rats were infused with vehicle control or PXR AS-ODNs 0, 24 and 44 hours before behavioral testing. Because effects of AS-ODNs can be transient, rats were administered three infusions at these time points before testing. The timing of infusions of AS-ODNs in the present study (at 0, 24, and 44 hours before testing) was based upon pilot studies using this technique and assessing reproductive behavior and evidence of reduction in PXR expression [28]. As well, the procedure aimed to have PXR knocked down before and during behavioral testing. Rats were only tested in one occasion, a half-hour after the last infusion. ODN chimeras were synthesized (capped oligos with remaining links unmodified) and desalted by Invitrogen Life Technologies (Carlsbad, CA). The vehicle for AS-ODN infusions was sterile saline. There were no differences were noted in rats that were administered saline vehicle or scrambled antisense ODN (5'CTCCGAAACGGACATCTGA3') as control infusions. As such, rats tested in these conditions were combined to form one control group. Irrespective of condition, all rats were infused with the same volume of infusate. For infusions, rats were gently held in one hand, resting on a table in the behavioral testing area. An infusion needle attached to PE tubing and a 5  $\mu$ l Hamilton syringe, both of which were filled with infusate, was carefully placed inside one cannula of the experimental rat. The experimenter depressed the syringe slowly (1  $\mu$ l/minute) and the infusion needle remained inside the cannula 1 minute. The process was then repeated for each rat's second cannula. Although it was not possible to directly determine the spread of the infusate in the present experiment, previous investigations have determined that such midbrain VTA infusions spread approximately 1 mm in all directions from infusion site and do not extend beyond the midbrain. Moreover, we have shown that

infusions of 3 $\alpha$ ,5 $\alpha$ -THP using the infusion methods described here to the substantia nigra also diffused approximately 1 mm in all directions and did not impinge upon the VTA [29].

**Behavioral Testing**—Behavioral data were collected from all rats except those used solely for PXR expression (n=22 from pilot experiment). Behavioral data were collected simultaneously using the Any-maze behavioral assessment computer program (for open field, elevated plus maze, and social interaction) or a digital video camera (for paced mating) and by experimenters. There is greater than 95% concordance between data “hand-collected” by experimenters and those data automatically recorded by Any-Maze (Stoelting Inc., Wood Lawn, IL). Any-Maze can be programmed to automatically record all possible indices in each task and digitally records a video of the performance of rats in each test. Rats were tested sequentially in the following tasks, in the order listed, using previously reported methods: open field [3,16], elevated plus maze [3,16], social interaction [3,16], and paced mating task [2,16,28]. Each cohort of rats was subjected to this entire battery of tasks.

### Open Field

The open field (76 × 57 × 35 cm) has a 48-square grid floor (6 × 8 squares, 9.5 cm/side), with 24 peripheral, 16 central, and 8 inner squares. Rats are placed in the open field, observed for 5 mins, while the number of entries made to the peripheral, central, and inner 8 squares (summed for total) is recorded [3,16]. The number of inner 8 square entries made by experimental groups compared to controls is utilized as an index of anti-anxiety behavior, while the total number of entries made by experimental groups compared to controls is an index of total motor behavior. Both of these measures in the open field are reported here as is the percentage of inner 8 entries to central entries.

### Elevated Plus Maze

The elevated plus maze consists of 2 arms (49 cm long, 10 cm wide), enclosed by walls 30 cm high (a.k.a. closed arms), and 2 exposed arms (a.k.a. open arms). The arms of the apparatus are elevated 50 cm off of the ground. Rats are placed at the junction of the open and closed arms. The number of entries, and amount of time spent on the open (compared to closed) arms, are recorded for 5 min [3,16]. The number of entries made, and time spent, in the open arms in experimental groups compared to controls is reported here as indices of anti-anxiety-like behavior. The percentage of open arm entries to total arm entries and the number of total arm entries are reported as general activity measures in the maze. The indices in the closed arms are not reported to avoid redundancy in the report as rats are either deemed to be in the open or closed arms.

### Social Interaction

The experimental subject and a conspecific rat (same-species, ovariectomized female) are placed in opposite corners of the open field. Time spent by the experimental rat engaging in social interaction (crawling over and under partner, sniffing of partner, following with contact, anogenital investigation, tumbling, and grooming) with the conspecific is recorded for 5 mins [3,16]. During this task, other performance measures, such as aggressive behavior (e.g. boxing, tail rattling, fighting, vocalizing), are recorded. These types of aggressive responses were not observed among any of the experimental subjects in this study.

### Paced Mating

A previously described protocol for paced mating was utilized [2,16,28]. The female’s pacing of male’s contacts for an ejaculatory series is evaluated in a chamber with a partition, which divides the chamber equally. Males are relegated to one side. The timing of female’s entries and exits to the male-side of the chamber, through a small hole, are recorded, as are

mounts, intromissions, and ejaculations by the male, so that return latencies, percent exits, inter-mount intervals, inter-intromission intervals, post-ejaculatory intervals, and frequency of each type of contact can be calculated. Proceptive (incidence of hopping, darting, and ear wiggling per total number of mounts), receptive (lordosis quotients, which is the percentage of total number of lordotic responses per total number of sexual contacts by the male, and lordosis ratings, which are the intensity of lordosis responses) aggression/rejection (aggression quotients; per total number of mounts) behaviors are recorded. Results from the most robust measures in the paced mating task (proceptivity, lordosis, and aggression quotients and lordosis ratings) are reported.

### Tissue Collection

All rats were rapidly decapitated. For rats that were behaviorally tested, this occurred immediately after testing. Whole brain and trunk blood were collected to measure steroid hormone levels in brain and plasma. Brains were immediately placed on dry ice and then stored at  $-80$  degrees C until dissections. For dissections, whole brains were briefly thawed on ice. From the whole brain, midbrain, hippocampus, striatum, and prefrontal cortex were dissected out. Dissected brain tissues were homogenized in 1ml ddH<sub>2</sub>O and 100  $\mu$ g of midbrain tissue was pipetted off and stored at  $-80$  degrees C for later western blot analyses.

During dissections, it was noted whether rats had accurate placement of infusions to the VTA. Figure 1 depicts a picture and drawing of correct and missed site infusions. Briefly, slices were made posterior and anterior of the midbrain region so that infusion site could be verified. From the ventral plane, the optic chiasm and pontine regions were utilized as posterior and anterior borders for the gross dissection of the midbrain, respectively. The lateral borders of the midbrain dissection were approximately 1.5 mm from midline of the brain and the dorsal border was below the cerebral aqueduct. Rats with missed infusion placement (n=20), based upon visual inspection, were not included in statistical analyses. Although missed control infusions did not produce robust differences in the pattern of behavior compared to intra-VTA infusions as did missed AS-ODN infusions, only data from rats with infusions that could be verified to be aimed at the midbrain VTA were included. As such, behavioral effects, PXR protein expression determined by western blotting, and hormone levels in the midbrain determined by radioimmunoassay of cycling rats administered control (diestrous n=11, proestrous n=13) or PXR AS-ODN (diestrous n=13, proestrous n=9) infusions to the VTA are described in the Results.

### qPCR methods

Standard qPCR methods were utilized on tissues from some rats. Tissues for qPCR were preserved in RNA later (Qiagen) to prevent degradation. Total RNA was isolated from tissue using the Qiagen RNeasy Micro Kit (Valencia, CA) according to the manufacturer's protocol. Reverse transcription was carried out using Oligo(dT)<sub>20</sub> and the Superscript III First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA). qPCR was performed using Bio-Rad SYBR Green Supermix (Hercules, CA) and the following gene-specific primers:  $\beta$ -actin forward (5'-GCT CGT CGT CGA CAA CGG CT-3'),  $\beta$ -actin reverse (5'-CAA ACA TGA TCT GGG TCA TCT TCT C-3'), PXR forward (5'-GCA TCC AGG ACA CGC ACC CC-3'), and PXR reverse (5'-GCC AGC ACG TCC CAC AGG CT-3'). Reactions were run on an Applied Biosystems 7900HT and analyzed using the comparative cycle time (DeltaDeltaCT) method (Applied Biosystems, Foster City, CA). DeltaDeltaCT values were normalized to the actin control and described as a percent change from control as an index of PXR mRNA expression.

## Protein Expression/Western Blot Analyses methods

A standard western blotting protocol was employed to assess PXR protein in some tissues. The western protocol included the following sample preparation for loading: 2.5  $\mu$ l of NuPAGE LDS (4 $\times$ ) sample buffer, 1  $\mu$ l of NuPAGE Reducing Agent (10 $\times$ ), 6.5  $\mu$ l of deionized water and 10  $\mu$ l of homogenized midbrain protein in 1 $\times$  sample buffer, which then were combined and loaded into NuPAGE Bis-Tris Mini Gels. Samples were run on NuPage 4–12% SDS Polyacrylamide gels with 1 $\times$  MOPS running buffer with one lane reserved for the protein ladder and one for the positive control (liver homogenate, and/or heart homogenate). After electrophoresis, protein was transferred to nitrocellulose using NuPAGE Transfer buffer (1 $\times$ ). The blots were blocked in 3% BSA or 5% milk solution. Western blots were probed with an unconjugated antibody to PXR overnight at 4C, (Santa Cruz Biotechnology, Santa Cruz, CA; 1:3000) and with the secondary antibody Goat Anti-Mouse IG (H+L) Horseradish Peroxidase Conjugate (1:1250) for 1 hour on a shaker at room temperature. (Bio-rad Hercules, CA). Results were visualized using DuoLuX Chemiluminescent/Fluorescent Substrate Kit for Peroxidase (Vector Laboratories, Burlingame, CA) and imaging on a ChemiDoc XRS (Bio-rad, Hercules, CA) and analyzed using ImageJ software.

## Radioimmunoassay for Steroid Hormones

Standard steroid extraction and radioimmunoassay techniques were employed to measure plasma and brain levels of E<sub>2</sub>, P<sub>4</sub>, DHP, and 3 $\alpha$ ,5 $\alpha$ -THP [16]. Steroids were extracted from tissues. Briefly, E<sub>2</sub>, P<sub>4</sub>, DHP, and 3 $\alpha$ ,5 $\alpha$ -THP were extracted from plasma with ether following incubation with water and 800 cpm of tritiated steroid. After snap-freezing twice, test tubes containing steroid and ether were evaporated to dryness in a heated savant vacuum centrifuge that removes organic solvents. Dried down tubes were then reconstituted with phosphate assay buffer to the original plasma volume. Progesterone, DHP, and 3 $\alpha$ ,5 $\alpha$ -THP were extracted from midbrain, hippocampus, striatum, and prefrontal cortex following homogenization of tissue with a pestle in 50% MeOH and 1% acetic acid. Samples were then centrifuged at 3000  $\times$  g in a centrifuge at 4 degrees C. Supernatant was chromatographed on Sepak-cartridges equilibrated with 50% MeOH and 1% acetic acid. Steroids were eluted with 50% and 100% MeOH. Tubes were placed in a savant and 100% MeOH was removed. Samples were reconstituted by adding 300  $\mu$ l assay buffer. Levels of steroids were determined with radioimmunoassay methods utilizing tritiated E<sub>2</sub> (specific activity 51.3 Ci/mmol), P<sub>4</sub> (specific activity 47.5 Ci/mmol), and 3 $\alpha$ ,5 $\alpha$ -THP (specific activity 65.0 Ci/mmol) were from Perkin Elmer (Boston, MA) and specific antibodies to E<sub>2</sub> (E#244, Dr. G.D. Niswender, Colorado State University, Fort Collins, CO), P<sub>4</sub> (P#337, Dr. G.D. Niswender, Colorado State University), and 3 $\alpha$ , 5 $\alpha$ -THP (#921412–5; Dr. Robert Purdy, Veterans Medical Affairs, La Jolla, CA). Tritiated steroids and antibodies were added to tubes containing reconstituted samples and incubated. Binding was terminated with addition of ice cold charcoal buffer to each assay tube. Binding was then determined by standard liquid scintillation spectroscopy. Standard liquid scintillation methods were used with correction for recovery and efficiency of the scintillation counter. Concentrations were determined for each sample based upon concurrent standard curves run for each of the assays.

## Statistical Analyses

In pilot experiments assessing PXR expression, descriptive statistics (mean  $\pm$  sem) were utilized to demonstrate PXR mRNA and protein expression in the midbrain of diestrous and proestrous rats. Descriptive, rather than inferential, statistics are reported on here given the small number of observations per group (n=2–5). Multiple, two-way analyses of variances (ANOVAs) were utilized to test our hypothesis that manipulating PXR in the midbrain would alter progestogens and behavior of cycling rats, particularly among proestrous rats.

To test this, we used two-way ANOVAs with 2 variables, each at 2 levels (estrous cycle phase (proestrous, diestrous) and PXR infusion condition (control, AS-ODN)) to determine effects on behavioral measures, endocrine levels, and PXR expression by Western blotting. The  $\alpha$  level for statistical significance was reached when  $p < 0.05$ . Fisher's *post hoc* tests were used to examine group differences.

## Results

### Pilot experiment-PXR expression in the midbrain

PXR mRNA and protein were expressed in the midbrain of rats. Proestrous rats demonstrated a  $23.7 \pm 13.0$  relative PXR expression change (relative to actin control) and diestrous rats demonstrated a  $0.9 \pm 0.5$  relative PXR expression change in the midbrain. Proestrous rats and diestrous rats administered PXR AS-ODNs to the VTA had lower relative expression changes compared to proestrous rats administered control infusions ( $1.1 \pm 0.9$  and  $9.9 \pm 9.8$ , respectively); albeit, there was large variability in these results and both diestrous rats did not show a clear reduction in PXR expression following AS-ODN infusions. PXR protein was expressed in the midbrain of rats. Proestrous rats demonstrated a  $2.8 \pm 1.5$  mean relative optical density of PXR (relative to actin bands) and diestrous rats demonstrated a  $1.0 \pm 0.2$  mean relative optical density of PXR in the midbrain.

### Midbrain PXR protein expression following PXR AS-ODN infusions to the VTA

There was a significant interaction of cycle condition and infusion condition [ $F(1, 42) = 7.31$ ,  $p < 0.01$ ] for mean relative density of PXR to actin control in the midbrain. Proestrous rats infused with PXR AS-ODNs, but not diestrous rats administered control or PXR AS-ODN infusions, had decreased PXR expression in the midbrain compared to proestrous rats (Figure 2).

### Open Field behavior following PXR AS-ODN infusions to the VTA

There was a significant difference for infusion condition [ $F(1, 42) = 7.09$ ,  $p < 0.01$ ] for behavior in the open field. Rats infused with PXR AS-ODNs made fewer inner 8 entries (index of anti-anxiety-like behavior) than did rats administered control infusions (Figure 3). There were no differences due to condition for total entries made in the open field (Table 1), but there was an effect of infusion condition for percentage of inner 8 to total entries in the open field [ $F(1, 42) = 4.40$ ,  $p < 0.04$ ]. When collapsing across cycle condition, compared to control infusions, PXR AS-ODN infusions reduced the percentage of inner 8 to total open field entries (Table 1).

### Elevated Plus Maze behavior following PXR AS-ODN infusions to the VTA

The effect of infusion condition for time spent the open arms of the elevated plus maze approached significance [ $F(1, 42) = 3.02$ ,  $p < 0.09$ ] (Figure 4), and there was a significant effect of infusion condition for open arm entries made [ $F(1, 42) = 4.65$ ,  $p < 0.04$ ; Table 1]. Rats infused with PXR AS-ODNs spent less time on the open arms of the plus maze and made fewer entries to the open arms than did rats administered control infusions. There were significant effects of infusion condition to alter total entries in the maze, such that rats administered PXR AS-ODNs made fewer total entries [ $F(1, 42) = 7.08$ ,  $p < 0.01$ ], but there were no differences in the percentage of open arm entries to total arm entries (Table 1).

### Social interaction following PXR AS-ODN infusions to the VTA

Proestrous rats spent less time interacting with a conspecific following infusions with PXR AS-ODN compared to rats administered control infusions, or diestrous rats administered



control or AS-ODN infusions. This was a similar pattern of effects as observed for paced mating measures, but this interaction did not reach statistical significance (Figure 5).

### Reproductive behavior following PXR AS-ODN infusions to the VTA

**Lordosis**—There was a significant interaction of cycle condition and infusion condition for lordosis quotients [ $F(1, 42) = 9.88, p < 0.01$ ]. Proestrous rats infused with PXR AS-ODNs, but not diestrous rats administered control or PXR AS-ODN infusions, had lower lordosis quotients (percentage of lordosis responses following a mount by a sexually-experienced male), compared to proestrous rats administered control infusions (Figure 6). There was a significant interaction of cycle condition and infusion condition for lordosis ratings [ $F(1, 47) = 3.89, p < 0.05$ ]. Proestrous rats infused with PXR AS-ODNs, but not diestrous rats administered control or PXR AS-ODN infusions, had lower lordosis ratings, compared to proestrous rats administered control infusions (Table 1).

**Proceptivity and Aggression**—A similar pattern was observed for proceptivity quotients. There was a significant interaction of cycle condition and infusion condition for proceptivity quotients [ $F(1, 47) = 6.56, p < 0.01$ ]. Proestrous rats infused with PXR AS-ODNs, but not diestrous rats administered control or PXR AS-ODN infusions, had lower proceptivity quotients, compared to proestrous rats administered control infusions (Table 1). There were no statistically significant effects for aggression quotients.

### Midbrain 3 $\alpha$ ,5 $\alpha$ -THP levels following PXR AS-ODN infusions to the VTA

There was a significant interaction among cycle condition and infusion condition [ $F(1, 42) = 7.36, p < 0.01$ ] for midbrain 3 $\alpha$ ,5 $\alpha$ -THP levels. Proestrous rats infused with PXR AS-ODNs, but not diestrous rats administered control or PXR AS-ODN infusions, had lower levels of midbrain 3 $\alpha$ ,5 $\alpha$ -THP compared to proestrous rats administered control infusions (Figure 7). This same pattern of significant differences due to PXR AS-ODN infusions was not noted for the other progestogens examined in the midbrain or other regions, or for 3 $\alpha$ , 5 $\alpha$ -THP levels in other regions, except for the striatum (see Table 2).

## Discussion

The present results supported our hypothesis that manipulating PXR expression in the VTA via AS-ODN infusions influences 3 $\alpha$ ,5 $\alpha$ -THP levels in the midbrain and behavior of proestrous rats. First, pilot studies indicated the expression of PXR mRNA and protein was determined in the midbrain of proestrous rats, and that VTA infusions of PXR AS-ODNs utilized in this study were effective in reducing PXR expression in the midbrain of proestrous rats. Compared to control infusions, infusions of PXR AS-ODNs to proestrous rats reduced expression of PXR protein in the midbrain, 3 $\alpha$ ,5 $\alpha$ -THP levels in the midbrain, and sexual receptivity of proestrous rats, with modest effects on anti-anxiety-like behavior in the elevated plus maze and open field. There were few effects of PXR AS-ODNs, compared to control infusions, on midbrain PXR, 3 $\alpha$ ,5 $\alpha$ -THP levels, or behavior of diestrous rats. These data demonstrate that knockdown of PXR expression in the midbrain of proestrous rats decreases 3 $\alpha$ ,5 $\alpha$ -THP levels, specifically in the midbrain, concomitant with attenuating sexual receptivity. Thus, 3 $\alpha$ -5 $\alpha$ -THP's effects in the midbrain for motivated, reproductively-relevant behaviors may require PXR for its formation.

The present study confirms and extends previous work on the importance of 3 $\alpha$ ,5 $\alpha$ -THP formation for motivated and reproductive behaviors. Natural elevations in, and infusions to the VTA of, 3 $\alpha$ ,5 $\alpha$ -THP enhance receptive and proceptive behaviors of rats [4,19,29–32]. Inhibiting TSPO, P450scc, 3 $\beta$ HSD, 5 $\alpha$ -reductase, or 3 $\beta$ HSD activity in the VTA of proestrous or hormone-primed rats attenuates lordosis, but this behavior is rescued by

increasing 3 $\alpha$ ,5 $\alpha$ -THP levels in the VTA [1,2,18,33]. For instance, in one study, proestrous rats were infused with vehicle, an inhibitor of biosynthesis (PK11195), and/or an inhibitor of metabolism (indomethacin), followed by a second infusion of vehicle or an enhancer of biosynthesis (FGIN 1–27), before behavioral assessment [23]. Inhibition of 3 $\alpha$ ,5 $\alpha$ -THP formation via biosynthesis or metabolism blockade decreased anti-anxiety behavior in the plus maze and open field, and sexual behavior, and midbrain levels of 3 $\alpha$ ,5 $\alpha$ -THP. These effects could be reversed with infusions of FGIN 1–27 [23]. In the present study, we demonstrate that knocking down PXR similarly reduces the percentage of lordosis responses of proestrous rats as was shown in these prior studies using pharmacological blockade of enzymes likely to be downstream of PXR. Moreover, knocking down PXR has similar effects to reduce the intensity of the lordosis response, as measured by lordosis ratings, as well as proceptive behavior of proestrous rats (see Table 1). Notably, a similar pattern of effects was observed in the social interaction task, albeit these effects were more variable and less robust than measures in the mating task. PXR knockdown in the midbrain attenuated anti-anxiety behavior in the open field by reducing the number of inner 8 entries made. PXR AS-ODNs to the midbrain had modest effects for anti-anxiety behavior in the elevated plus maze, as demonstrated by a non-significant reduction in the time spent on the open arms and a significant reduction in the entries made to the open arms. Some of these effects in the open field and elevated plus maze may be related to reductions in motor activity following PXR AS-ODN infusions as there were smaller percentages of central or open arm entries to total entries, as well as reductions in central entries and open arm time/entries, in the open field and elevated plus maze, respectively. It may also be that these modest effects on anti-anxiety-like behavior in the elevated plus maze and open field are related to reductions in 3 $\alpha$ ,5 $\alpha$ -THP being more robust in the midbrain, than in brain regions traditionally considered to be involved in anxiety-related behaviors, such as the hippocampus. Because rats were tested in a battery, it is unclear whether effects on reproductive behavior were secondary to changes in the other behavioral tasks assessed (social interaction, open field, and elevated plus maze). It can be argued that the most consistent effects of PXR knockdown in this study were the reduction of lordosis and midbrain 3 $\alpha$ ,5 $\alpha$ -THP levels. Additionally, of interest is whether 3 $\alpha$ ,5 $\alpha$ -THP replacement would attenuate these effects by rescuing the loss of 3 $\alpha$ ,5 $\alpha$ -THP due to PXR knockdown. A related consideration in interpreting the present data is that PXR is a transcription factor and could have actions on other genes that may be related, or not, to 3 $\alpha$ ,5 $\alpha$ -THP formation. Notably, others have demonstrated that ovarian expression of the CYP enzyme, P450<sub>scc</sub>, is elevated preceding, and coincident with, onset of sexual receptivity in rats, and declines in later stages of the estrous cycle [34]. In the present study, these prior results were extended with the observation that PXR expression in the midbrain was higher among rats in proestrus than in diestrus and this coincided with increased 3 $\alpha$ ,5 $\alpha$ -THP levels in this region. Thus, these prior and present findings suggest that 3 $\alpha$ ,5 $\alpha$ -THP formation, which may require PXR, in the midbrain VTA is important for appetitive (proceptive behavior), as well as consummatory (lordosis), aspects of mating behavior.

In addition to assessing the aforementioned functional effects, the present study extends what is known about the localization and function of PXR beyond liver metabolism. Indeed, PXR is most often studied in the liver, where it exists in relatively high concentrations [35], and differences in liver expression have been shown across pregnancy and the post-partum period of mice coincident with clear changes in P<sub>4</sub> concentrations [36]. Beyond the liver, we have demonstrated in the present study among proestrous rats that PXR is expressed at the gene, RNA, and protein level in the midbrain, is a target for motivated behaviors and is involved in the reproductive circuit [1,12,35]. Other reports, using RT-PCR and Western blotting, have shown that PXR is expressed in the central nervous system. In support, PXR mRNA and protein is expressed in the rabbit cortex, midbrain, cerebellum [35], rat brain capillaries [37], and various regions of the human brain [38]. Higher gestational levels of

3 $\alpha$ ,5 $\alpha$ -THP are associated with greater whole brain expression of PXR mRNA in offspring [36]. Moreover, in the present study, a cycle-dependent effect of PXR expression was revealed with rats in proestrous having higher expression than those in diestrous. The pilot results using qRT-PCR and Western blotting in and the Western blotting in behaviorally-tested rats confirmed what we have observed in a microarray study, using Affymetrix GeneChip Rat 230 2.0 arrays, assessing mRNA changes across proestrous mated rats and those that were not mated [30]. Although not specifically reported in this publication, we observed in these analyses that PXR mRNA was expressed in grossly-dissected out midbrain [30]. Of interest is the site-specificity for these effects. Pilot studies demonstrate cyclical differences in PXR expression in the midbrain. The effects of PXR AS-ODNs to the midbrain produced the clearest decrements in 3 $\alpha$ ,5 $\alpha$ -THP formation; albeit, there is some indication of a similar pattern in the striatum and a more variable and non-significant pattern for hippocampal levels of 3 $\alpha$ ,5 $\alpha$ -THP to be reduced in proestrous rats following knock down of PXR in the midbrain. A different pattern of effects was observed among rats that were administered PXR AS-ODNs infusions outside of the VTA. Indeed, these rats did not have lower midbrain PXR expression, lower levels of 3 $\alpha$ ,5 $\alpha$ -THP in the midbrain, decreased anxiety-like behavior and sexual receptivity as was more clearly observed among rats administered PXR AS-ODNs infusions to the VTA. As these differences were most notable for 3 $\alpha$ ,5 $\alpha$ -THP levels in the midbrain and a different pattern was observed for infusions outside of the midbrain, the data suggest site-specificity of the experimental effects of the PXR AS-ODNs in the midbrain where the infusions were aimed. Moreover, the reduction in PXR expression in the midbrain seems to be most evident in the area closest to the infusion site in the VTA [28]. Although previous systematic investigations of the diffusion of VTA infusions do not suggest spread beyond the midbrain [29], we do not know if midbrain VTA infusions utilized here spread to certain hypothalamic nuclei, such as mammillary, parabrachial or interpeduncular, which are near to VTA. There was a non-significant decrease in 3 $\alpha$ -THP levels in the grossly-dissected out hypothalamus among proestrous rats after PXR AS-ODN infusions (from 13 $\pm$ 4.3 in controls to 3.5 $\pm$ 0.7 in PXR AS-ODN infused rats), which may suggest that there was spread of the AS-ODNs to the hypothalamus, or that midbrain manipulations alter hypothalamus levels of 3 $\alpha$ ,5 $\alpha$ -THP. We are presently investigating these site-specific effects further in follow-up studies. Thus, these data substantiate future investigations of the functional effects of PXR in the midbrain of females over reproductive cycles.

Cycle-dependent effects of PXR and/or catalyzing enzyme expression indicate modulation via endogenous hormonal milieu. Progesterone plays an important role in modulating estrous cyclicity among rodents [39] and E<sub>2</sub> also influences circadian timing of estrous cycle-promoting factors [40]. It is important to understand the extent to which estrogens and/or progestogens contribute to these effects involving PXR. Others have found that systemic administration of E<sub>2</sub> to OVX/ADX rats enhances 3 $\beta$ -HSD activity and mRNA expression in hypothalamus [41]. 3 $\alpha$ ,5 $\alpha$ -THP levels in the midbrain are particularly dynamic and change in response to ovarian secretion of E<sub>2</sub> and P<sub>4</sub>, and with mating stimuli. In order for rodents to be sexually-receptive, ovarian secretion or exogenous administration of E<sub>2</sub> is necessary [1,42,43]. E<sub>2</sub> increases the formation of central progestogens, the activity of the 5 $\alpha$ -reductase enzyme, and formation of 3 $\alpha$ ,5 $\alpha$ -THP [44]. Further increases in receptive behaviors are produced by ovarian secretion of progestogens or exogenous administration of progestogens, which increases midbrain levels of 3 $\alpha$ ,5 $\alpha$ -THP over that of diestrous, OVX, or OVX, E<sub>2</sub>-primed rodents [1]. Although the present study showing little to no effects of PXR manipulations to diestrous rats suggests that PXR may be involved in progestogens-, rather than E<sub>2</sub>-, facilitated lordosis, ongoing studies are systematically addressing this question.

Another question of interest is the influence of mating itself on midbrain PXR and 3 $\alpha$ ,5 $\alpha$ -THP levels in the present study. As discussed above, enhancing 3 $\alpha$ ,5 $\alpha$ -THP levels in the midbrain VTA facilitates sexual receptivity of rodents. Moreover, engaging in paced mating can enhance biosynthesis of 3 $\alpha$ ,5 $\alpha$ -THP in midbrain of rats [32,45]. Such enhancement in 3 $\alpha$ ,5 $\alpha$ -THP levels is not observed in standard mating paradigms. There are important consequences of this mating-induced enhancement, including improving fertility and fecundity [31] and, increases in 3 $\alpha$ ,5 $\alpha$ -THP following mating in other regions (hippocampus, hypothalamus, cortex, striatum) that may underlie progestogens' mediation of anxiety behavior and motivation [4,19,32,46,47]. In addition to these dynamic neuroendocrine changes and enhancement in pregnancy outcomes, paced mating can produce a conditioned place preference [2,48,49], which further supports progestogens' role in motivated processes. Of interest in the present study is that the largest differences in progestogen concentrations outside the midbrain were those that occurred in the striatum for 3 $\alpha$ ,5 $\alpha$ -THP. These data suggest that 3 $\alpha$ ,5 $\alpha$ -THP and PXR may be factors in this well-known circuitry of the VTA and striatum for motivated behaviors. A follow-up question of interest would be the extent to which these effects are bidirectional, with PXR manipulations in the striatum altering 3 $\alpha$ ,5 $\alpha$ -THP in the midbrain. Another interpretation of these data is that PXR in the brain alters peripheral steroid output which in turn affects brain levels of 3 $\alpha$ ,5 $\alpha$ -THP, rather than a direct effect on 3 $\alpha$ ,5 $\alpha$ -THP synthesis from cholesterol in the VTA. However, a lack of changes in peripheral levels of 3 $\alpha$ ,5 $\alpha$ -THP and its precursors, P<sub>4</sub> and DHP, in the present study, is not supportive of this notion.

Another consideration is the extent to which 3 $\alpha$ ,5 $\alpha$ -THP may alter PXR. 3 $\alpha$ ,5 $\alpha$ -THP is an endogenous positive activator of the PXR [36]. Enhancing gestational levels of 3 $\alpha$ ,5 $\alpha$ -THP can increase whole brain expression of PXR mRNA in offspring [36]. With PXR knockdown in the present study, there was lower 3 $\alpha$ ,5 $\alpha$ -THP production in the midbrain, but there should still be responses of rats to 3 $\alpha$ ,5 $\alpha$ -THP administration to the midbrain. We are currently investigating this question. Moreover, a battery of tasks was utilized in this study, as we were interested in the extent to which manipulating PXR would alter these reproduction-related behaviors and 3 $\alpha$ ,5 $\alpha$ -THP levels in the same subject. However, it can be difficult to interpret effects of manipulations on any one measure when such a battery of tasks is utilized. Future studies will investigate specific effects in each of these tasks for 3 $\alpha$ ,5 $\alpha$ -THP synthesis. Indeed, the extent to which PXR contributes to mating-related biosynthesis of 3 $\alpha$ ,5 $\alpha$ -THP in the VTA as well as other areas involved in motivated and reproduction-related behaviors, such as the striatum/nucleus accumbens, is of continued interest. Further investigation of downstream receptor targets of 3 $\alpha$ ,5 $\alpha$ -THP is warranted. For example, clinical efficacy of flibanserin, a 5-HT<sub>1A</sub> agonist and 5-HT<sub>2A</sub> antagonist, in treating hypoactive sexual desire disorder among women, and other recent studies in female rodents and primates administered this compound, support a role of manipulating these serotonin receptors for receptivity [50–55]. Moreover, acute and repeated dosing of flibanserin to female rats had greater effects to increase levels of dopamine and norepinephrine, compared to serotonin, in areas such as the prefrontal cortex, nucleus accumbens and medial preoptic area [56]. How 3 $\alpha$ ,5 $\alpha$ -THP, which has well-characterized effects through neurotransmitter targets, such as GABA, dopamine, and glutamate in the VTA for female rodent receptivity [1, 30], may potentially be involved in some of these actions of flibanserin, likely downstream of PXR, is an interesting question for future studies.

## Conclusions

The present results support the notion that PXR, a promiscuous nuclear receptor, is localized to the midbrain VTA and is required for proestrous increases in 3 $\alpha$ ,5 $\alpha$ -THP levels. In the midbrain VTA, 3 $\alpha$ ,5 $\alpha$ -THP plays a role to influence motivated behaviors, such as mating.

The functional effects of PXR in the midbrain are of continued interest and are currently being investigated further in the laboratory.

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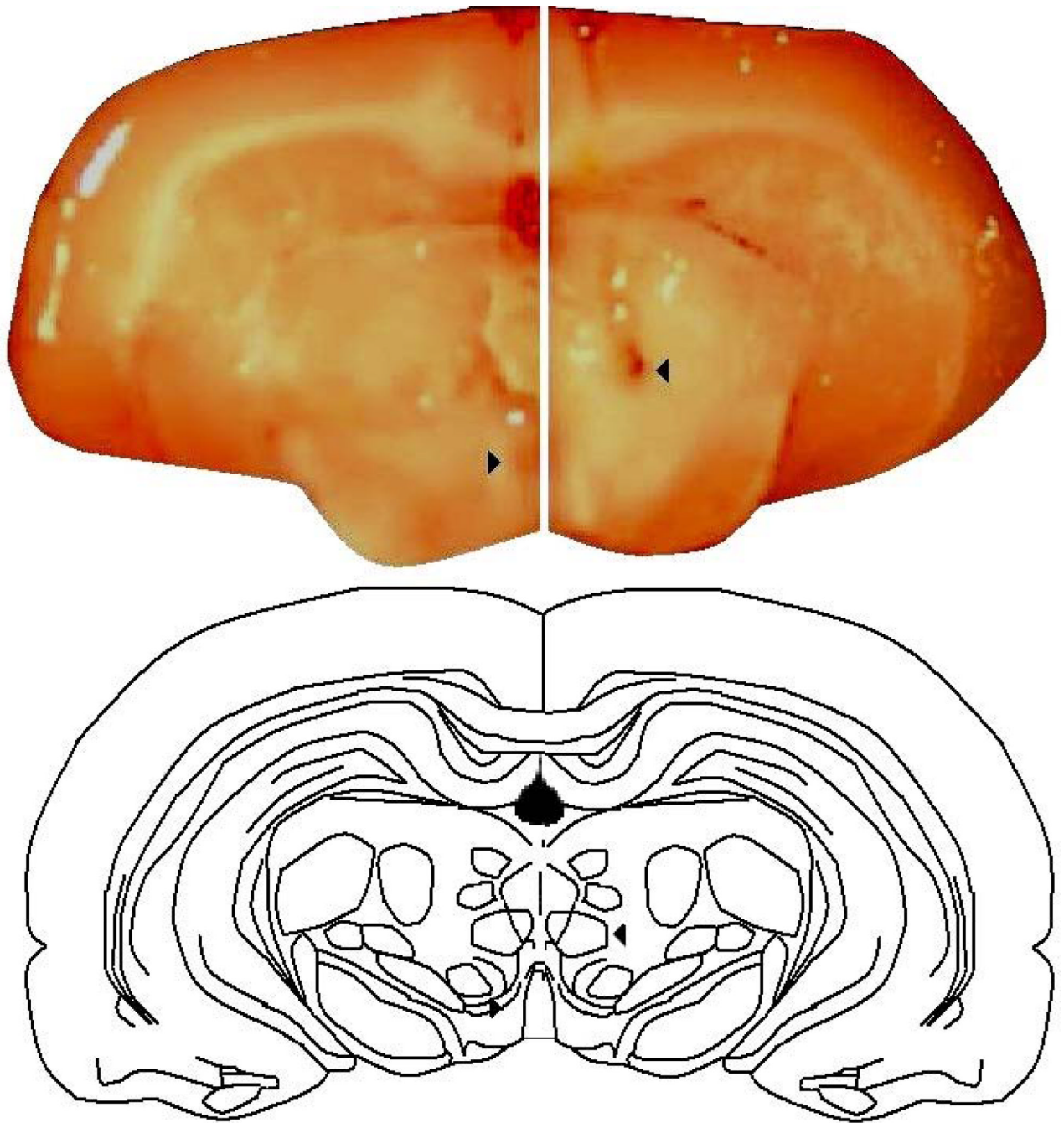
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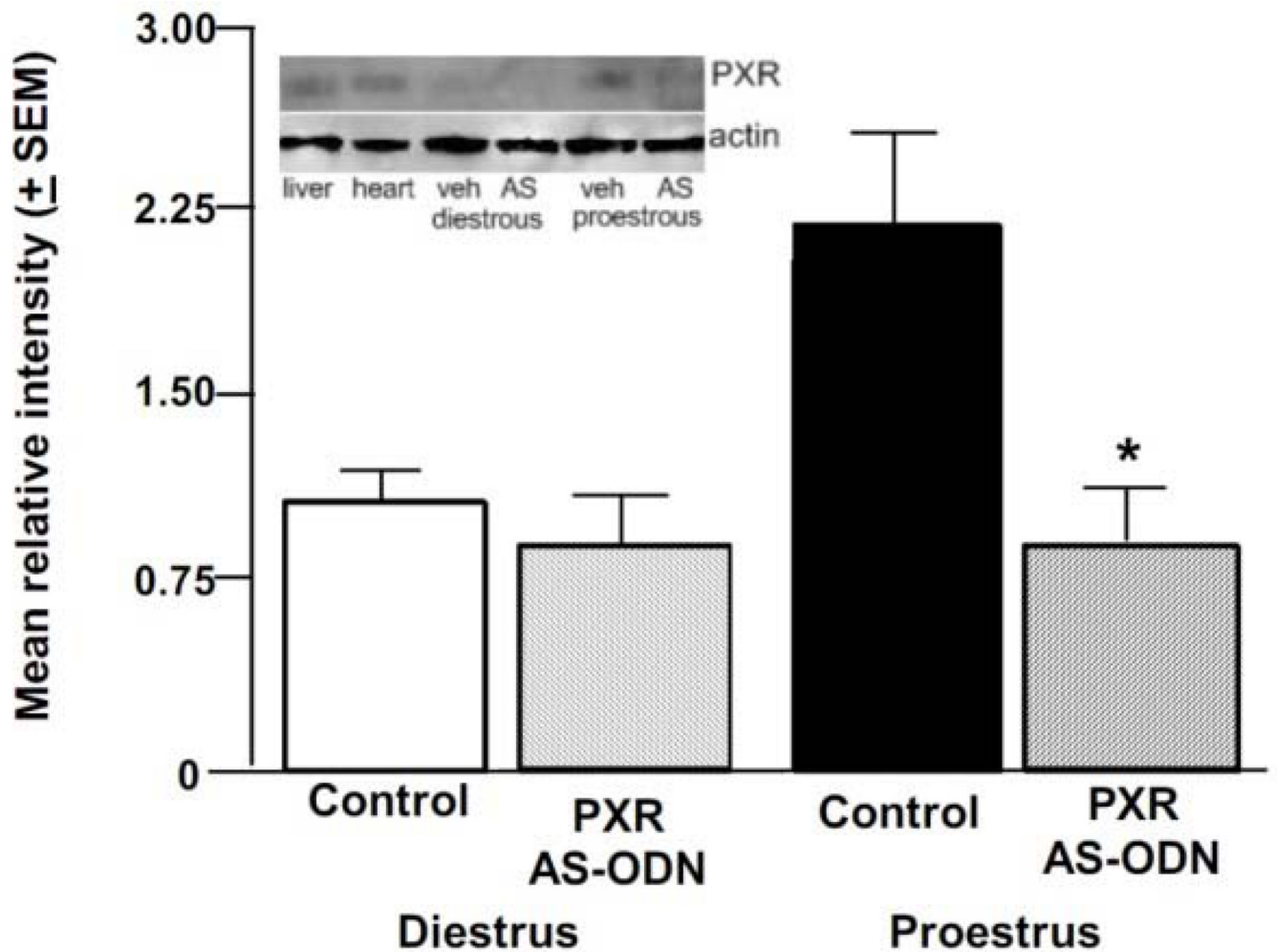
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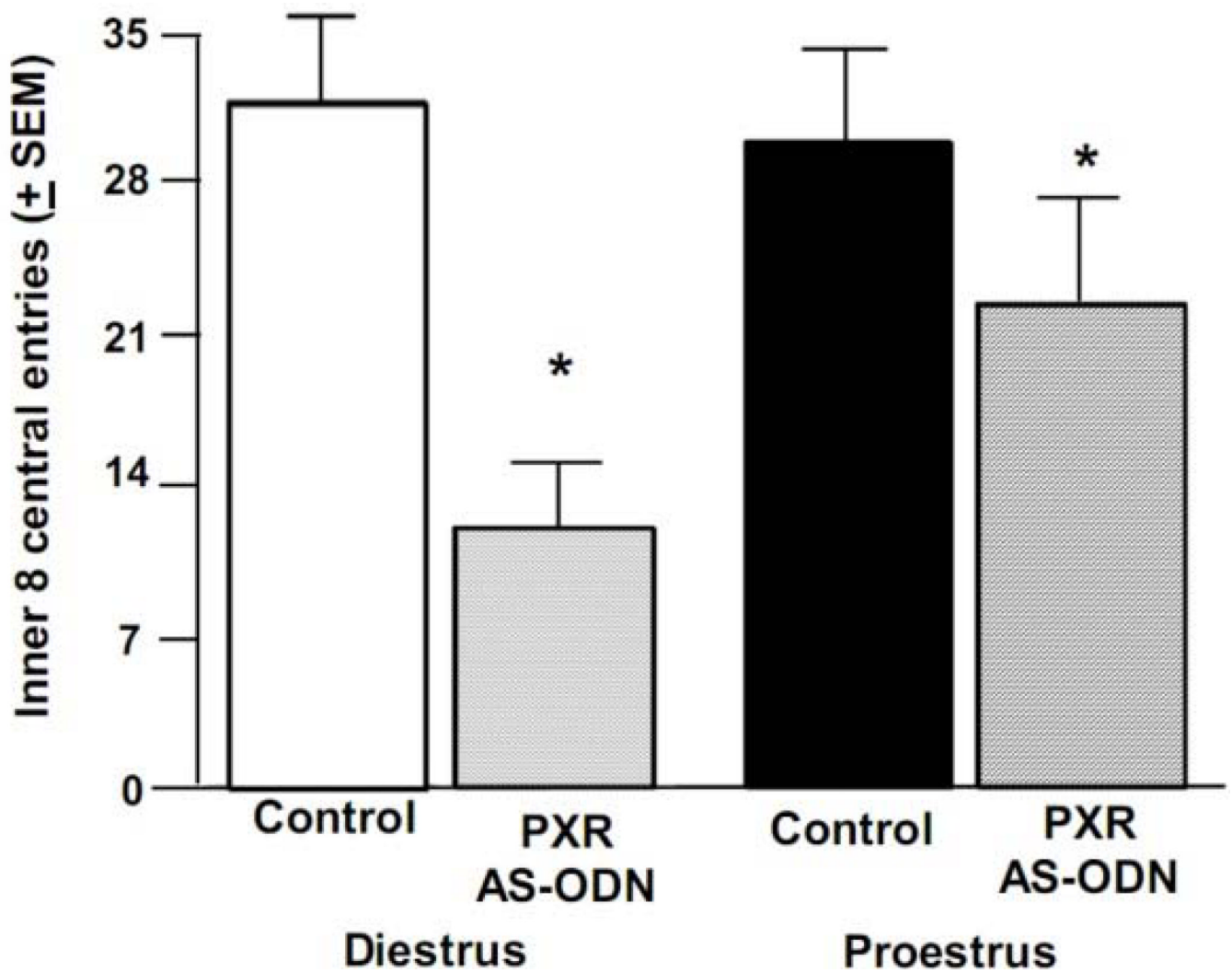
**Figure 1.**

Brains were visually inspected during dissection to verify that infusions were to the midbrain (shown on left, indicated by arrowhead) or outside of the midbrain (shown on right, indicated by arrowhead). Data from 20 rats that had infusions outside of the midbrain were not included in statistical analyses.

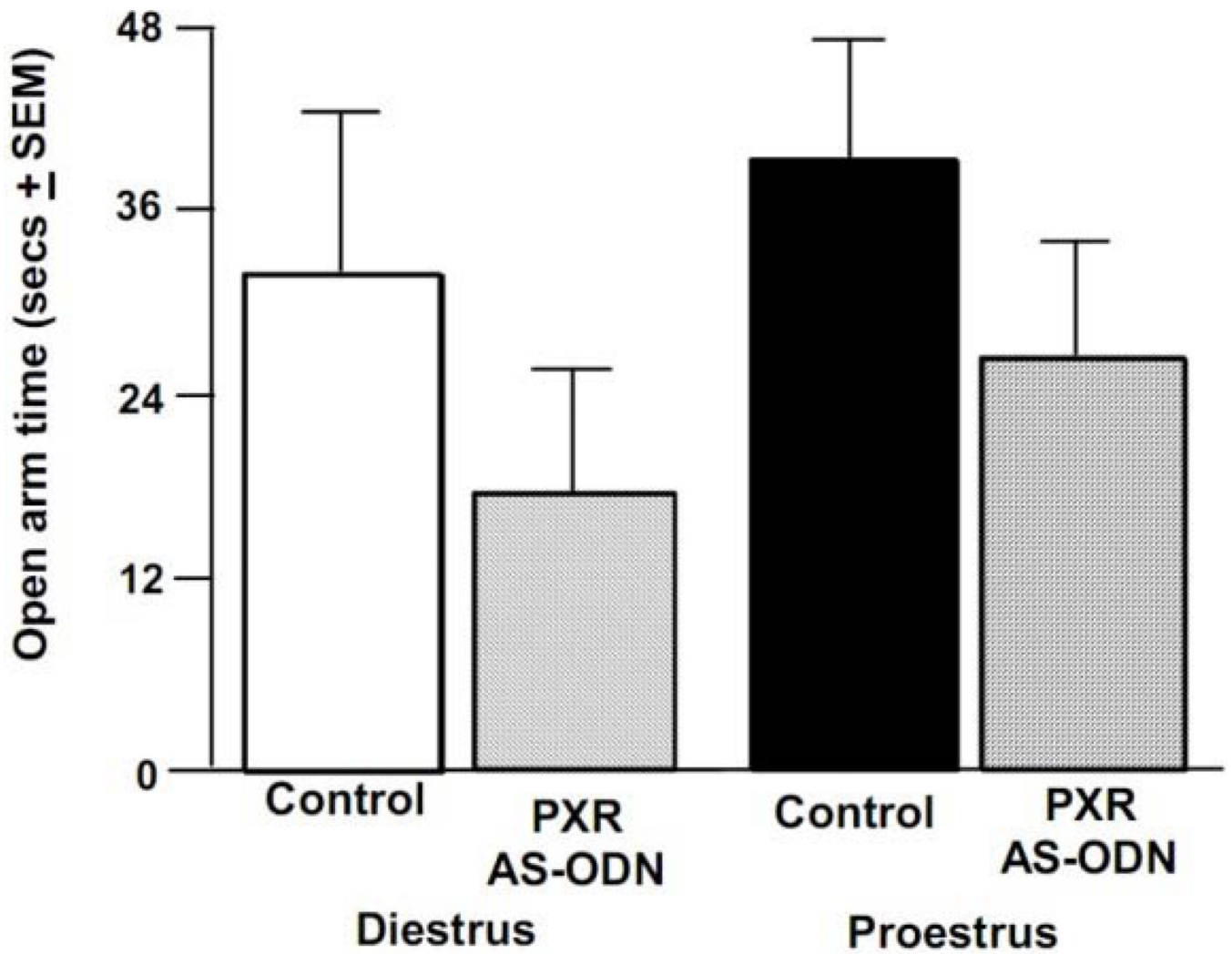


**Figure 2.**

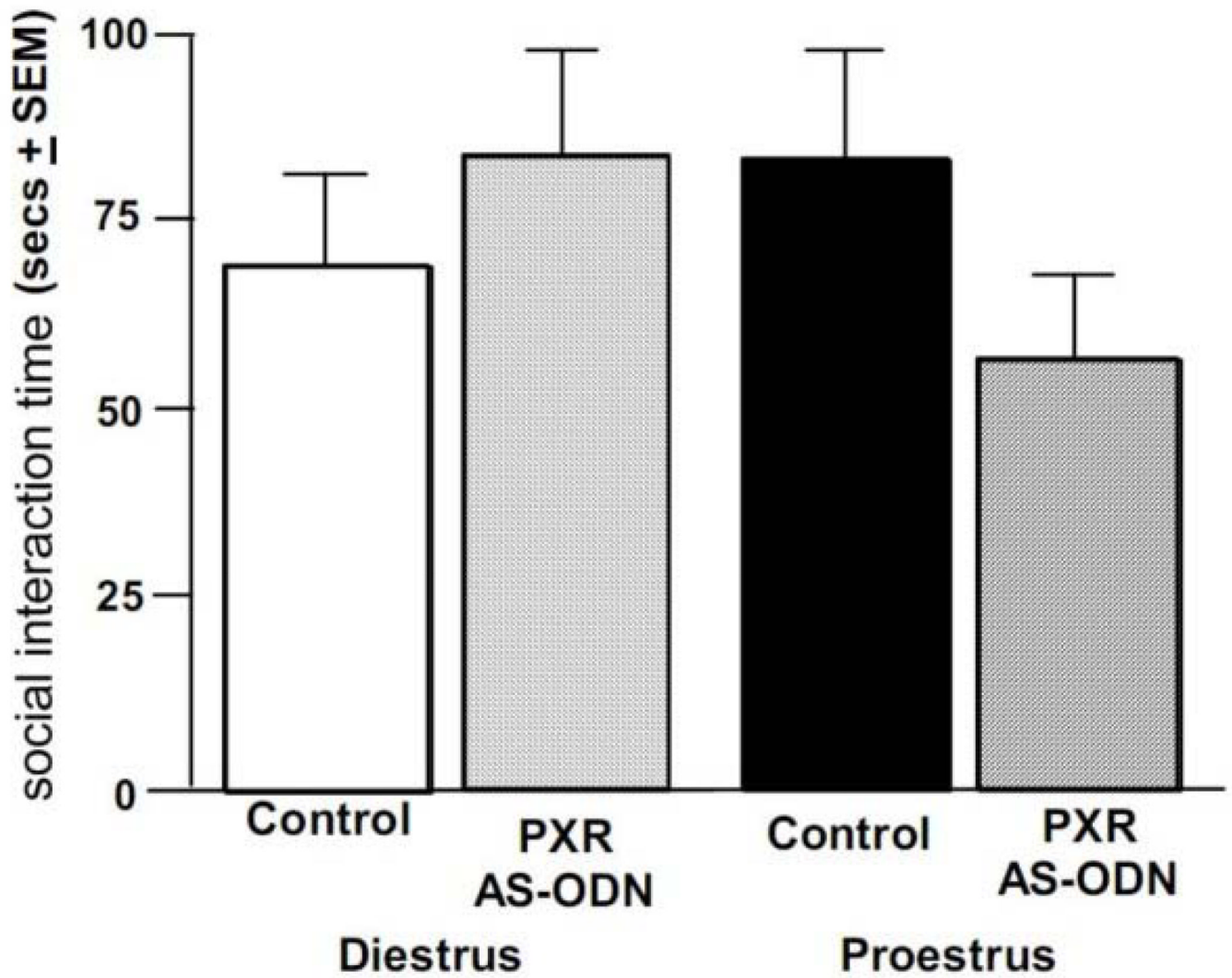
Depicts mean (+SEM) relative optical density of midbrain PXR bands to actin bands on the same blots of cycling rats administered vehicle control (diestrus n=11, proestrus n=13) or PXR AS-ODNs (diestrus n=13, proestrus n=9) infusions that were behaviorally-tested and had plasma and brain levels of steroids measured. Inset depicts representative picture of blot run for this experiment. In each blot, lanes were run with positive controls (liver and heart) and experimental conditions. \* indicates a reduction due to PXR AS-ODN, compared to control, infusions of proestrus rats ( $P < 0.05$ ).



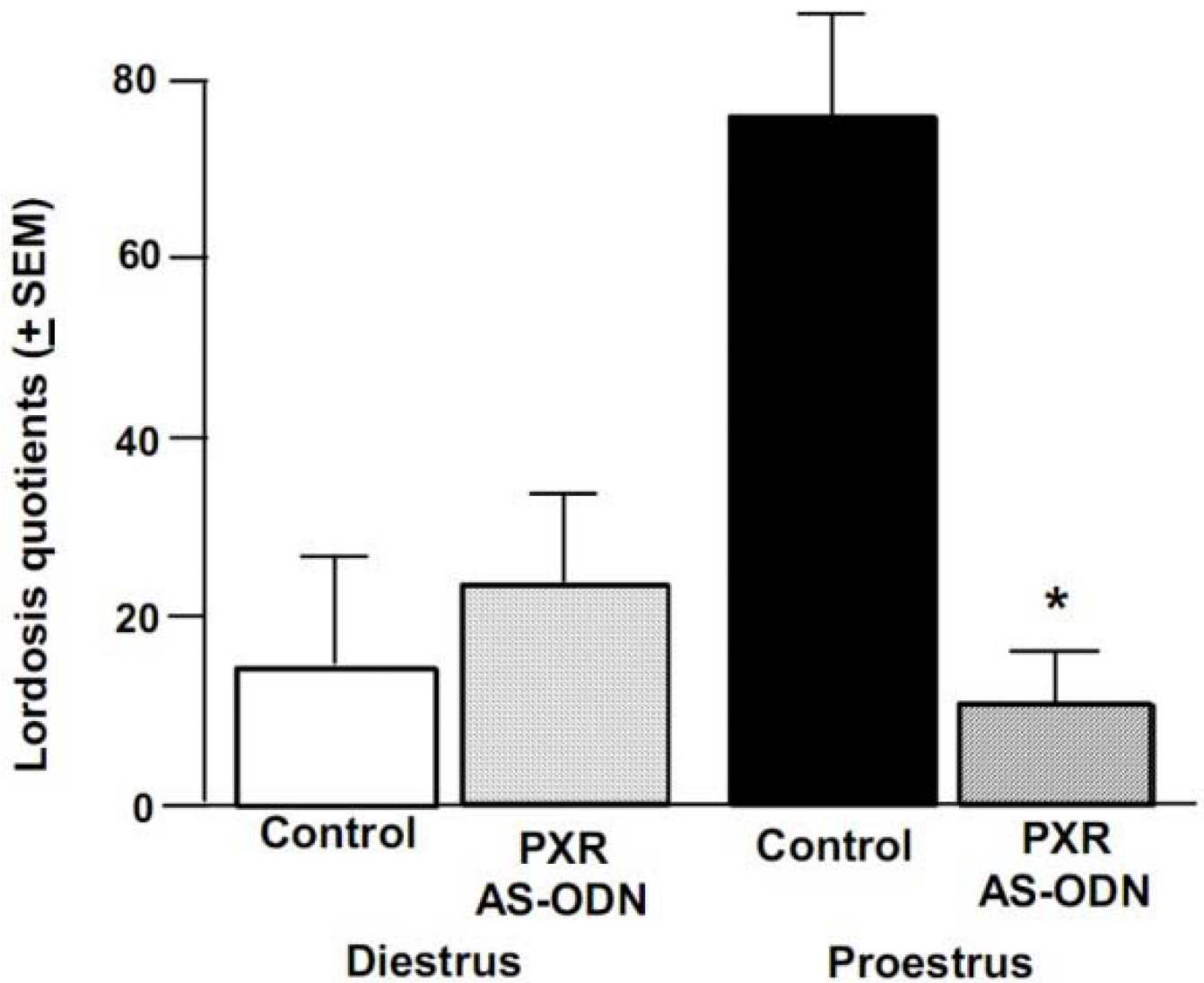
**Figure 3.** Depicts mean (+ SEM) entries to inner 8 squares in the open field of cycling rats administered vehicle control (diestrus n=11, proestrous n=13) or PXR AS-ODNs (diestrus n=13, proestrous n=9) infusions. \* indicates a reduction due to PXR AS-ODN, compared to control, infusions ( $p < 0.05$ ).



**Figure 4.** Depicts mean (+ SEM) open arm time in the elevated plus maze of cycling rats administered vehicle control (diestrus n=11, proestrus n=13) or PXR AS-ODNs (diestrus n=13, proestrus n=9) infusions.

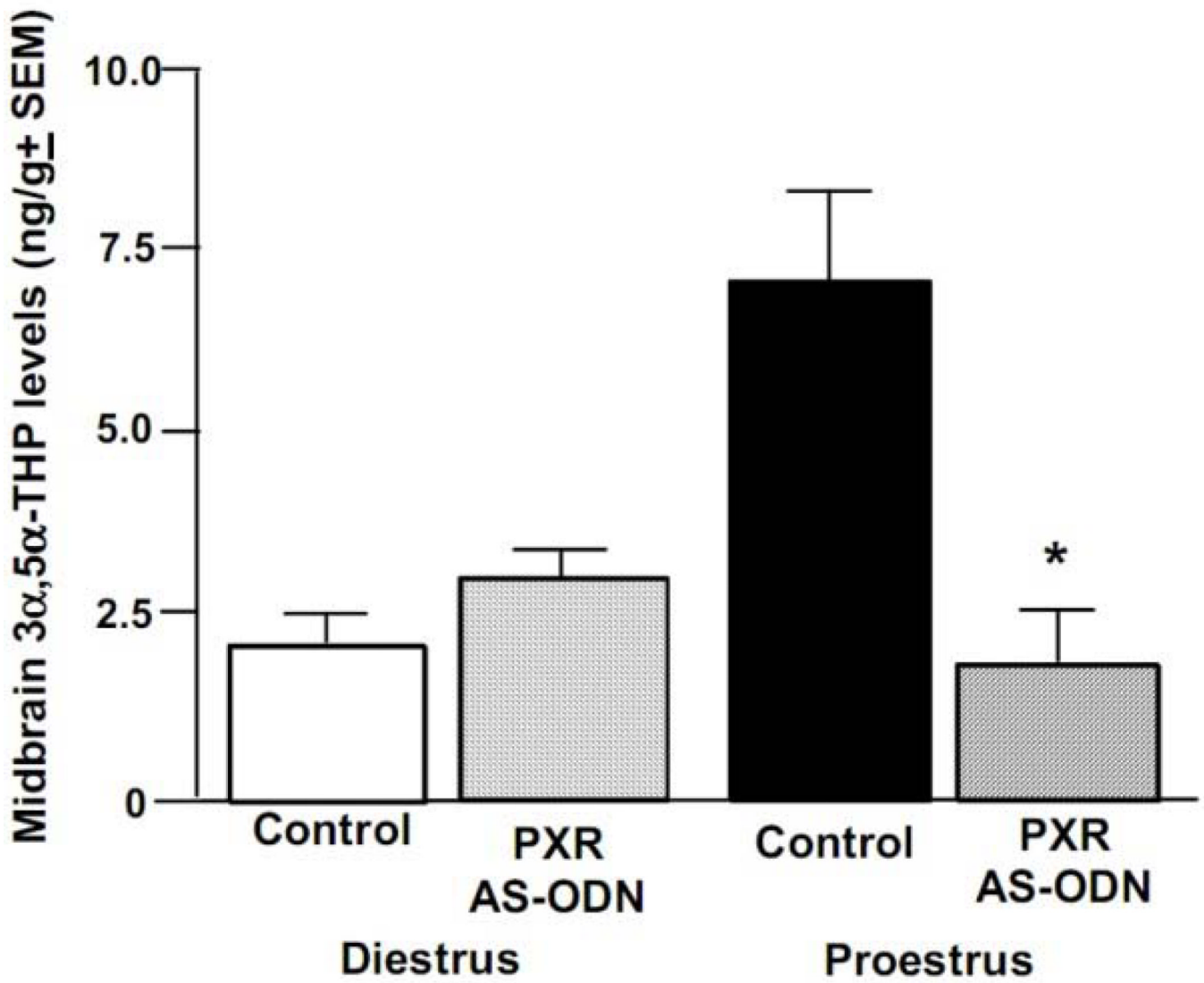


**Figure 5.** Depicts the mean (+S.E.M.) time spent in social interaction of cycling rats administered vehicle control (diestrus n=11, proestrous n=13) or PXR AS-ODNs (diestrus n=13, proestrous n=9) infusions.



**Figure 6.**

Depicts the mean (+S.E.M.) lordosis quotients of cycling rats administered vehicle control (diestrus n=11, proestrous n=13) or PXR AS-ODNs (diestrus n=13, proestrous n=9) infusions. \* indicates a reduction due to PXR AS-ODN, compared to control, infusions among proestrous rats ( $P < 0.05$ ).



**Figure 7.**

Depicts mean (+SEM) 3α,5α-THP levels of cycling rats administered vehicle control (diestrus n=11, proestrous n=13) or PXR AS-ODNs (diestrus n=13, proestrous n=9) infusions. \* indicates a reduction due to PXR AS-ODN, compared to control, infusions among proestrous rats (P<0.05).

**Table 1**

Behavioral measures of diestrous or proestrous rats administered control or pregnane xenobiotic receptor (PXR) antisense oligodeoxynucleotide (AS-ODN) infusions to the midbrain ventral tegmental area.

Behavioral Measures	Condition			
	Diestrous control infusion to VTA	Diestrous PXR AS-ODN infusion to VTA	Proestrous control infusion to VTA	Proestrous PXR AS-ODN infusion to VTA
Lordosis ratings	0.4 ± 0.2	0.5 ± 0.3	1.5 ± 0.3	0.5 ± 0.3 <sup>^</sup>
Proceptivity quotients	9.1 ± 9.1	16.7 ± 10.3	50.4 ± 10.1	8.3 ± 5.9 <sup>^</sup>
Aggression quotients	41.1 ± 10.8	42.5 ± 9.9	42.3 ± 9.4	37.8 ± 12.7
Open arm entries in the plus maze	4.5 ± 1.2	2.2 ± 1.0 <sup>*</sup>	4.8 ± 1.0	2.6 ± 0.6 <sup>*</sup>
Total arm entries in the plus maze	16.1 ± 1.8	10.6 ± 1.7 <sup>*</sup>	14.5 ± 1.6	11.1 ± 1.5 <sup>*</sup>
Percentage of open arm entries to total entries in the plus maze	25.0 ± 5.4	15.9 ± 5.2	28.2 ± 4.7	23.6 ± 5.1
Total open field entries	385.2 ± 34.9	301.8 ± 22.3	343.2 ± 20.4	354.3 ± 106.7
Percentage of open field inner 8 central entries to total entries	7.9 ± 1.1	4.5 ± 0.8 <sup>*</sup>	8.3 ± 0.9	7.2 ± 1.6 <sup>*</sup>

\* indicates significant difference for PXR infusion condition compared to control infusions (but no effect of cycle phase for these results).

<sup>^</sup> indicates significant interaction between estrous cycle and PXR infusion condition.



**Table 2**

Plasma levels of estradiol (E<sub>2</sub>) and plasma and brain levels of progesterone (P), dihydroprogesterone (DHP), and 3 $\alpha$ ,5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (3 $\alpha$ ,5 $\alpha$ -THP) of diestrous or proestrous rats administered control or pregnane xenobiotic receptor (PXR) antisense oligodeoxynucleotide (AS-ODN) infusions to the midbrain ventral tegmental area. Please note that midbrain levels of 3 $\alpha$ ,5 $\alpha$ -THP are included in Figure 7.

Steroid levels	Condition			
	Diestrous control (n=11)	Diestrous PXR AS-ODN (n=13)	Proestrous Control (n=13)	Proestrous PXR AS-ODN (n=9)
Plasma				
E <sub>2</sub> (pg/ml)	8.6 ± 3.9	13.7 ± 6.7	27.6 ± 9.1	21.6 ± 7.4
P <sub>4</sub> (ng/ml)	13.5 ± 3.2	12.5 ± 3.5	8.6 ± 1.3	10.9 ± 3.3
DHP (ng/ml)	25.5 ± 2.1	34.9 ± 5.2	27.4 ± 4.5	22.9 ± 4.5
3 $\alpha$ ,5 $\alpha$ -THP (ng/ml)	55.3 ± 16.7	70.7 ± 17.8	71.5 ± 20.0	72.5 ± 14.5
Cortex				
P <sub>4</sub> (ng/mg)	2.9 ± 0.5	3.9 ± 0.9	3.5 ± 0.6	4.0 ± 0.9
DHP (ng/mg)	15.7 ± 2.5	12.4 ± 2.2	14.0 ± 3.0	19.3 ± 3.8
3 $\alpha$ ,5 $\alpha$ -THP (ng/mg)	4.2 ± 1.4	4.9 ± 1.1	3.5 ± 0.7	6.9 ± 2.1
Hippocampus				
P <sub>4</sub> (ng/mg)	3.2 ± 0.8	3.9 ± 0.7	4.1 ± 0.5	2.9 ± 0.7
DHP (ng/mg)	7.0 ± 1.4	9.4 ± 1.2	10.2 ± 1.6	8.6 ± 2.2
3 $\alpha$ ,5 $\alpha$ -THP (ng/mg)	4.7 ± 1.3	5.6 ± 1.7	21.9 ± 14.2	3.1 ± 1.0
Striatum				
P <sub>4</sub> (ng/mg)	1.7 ± 0.5	2.3 ± 0.6	6.0 ± 1.8	1.2 ± 0.3 <sup>^</sup>
DHP (ng/mg)	3.6 ± 0.8	4.1 ± 0.7	5.2 ± 0.6	3.8 ± 0.9
3 $\alpha$ ,5 $\alpha$ -THP (ng/mg)	2.1 ± 1.2	1.5 ± 0.7 <sup>*</sup>	3.7 ± 1.0	0.4 ± 0.1 <sup>*</sup>
Hypothalamus				
P <sub>4</sub> (ng/mg)	5.3 ± 1.3	3.6 ± 1.1	3.7 ± 0.8	3.9 ± 1.0
DHP (ng/mg)	15.4 ± 2.9	11.1 ± 1.5	11.4 ± 1.8	10.7 ± 2.4
3 $\alpha$ ,5 $\alpha$ -THP (ng/mg)	11.4 ± 2.9	11.1 ± 6.2	13.0 ± 4.3	3.5 ± 0.7
Midbrain				
P <sub>4</sub> (ng/mg)	12.2 ± 3.6	8.3 ± 1.6	13.1 ± 3.3	12.3 ± 2.7
DHP (ng/mg)	3.1 ± 0.5	5.8 ± 1.3	6.6 ± 1.0 <sup>**</sup>	6.4 ± 0.9 <sup>**</sup>
3 $\alpha$ ,5 $\alpha$ -THP	Please see Figure 7.			

<sup>\*</sup> indicates significant difference compared to control infusions [ $F(1, 42) = 4.62, p < 0.04$ ].

<sup>\*\*</sup> indicates significant difference compared to diestrous rats [ $F(1, 42) = 3.81, p < 0.05$ ].

<sup>^</sup> indicates significant interaction between estrous cycle and PXR infusion condition [ $F(1, 42) = 6.08, p < 0.02$ ].