ESTROGEN AND PROGESTERONE PROTECT AGAINST THE INHIBITORY EFFECTS OF RESTRAINT STRESS

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BY

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Date

To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Stacy N. White entitled "Estrogen and Progesterone Protect Against the Inhibitory Effects of Restraint Stress." I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

Lynda Uphouse
Lynda Uphouse, Major Professor

We have read this dissertation and recommend its acceptance:

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Accepted:

Jennifer Martin
Dean of the Graduate School

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ABSTRACT

ESTROGEN AND PROGESTERONE PROTECT AGAINST THE INHIBITORY EFFECTS OF RESTRAINT STRESS

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The effects of restraint stress on lordosis behavior were examined in ovariectomized (ovx), hormone-primed rats and in proestrous rats. In the first experiment progesterone (P) dose-dependently reduced the female rat's response to restraint. Ovx rats were primed with 10 μg estradiol benzoate (EB) followed 48 hr later with various doses of progesterone or sesame seed oil (oil). At 5 min after restraint, rats that were given oil or 2.5, 5.0, or 10 μg progesterone and restrained for 5 min showed a decline in lordosis behavior. By 10 min after restraint lordosis had increased; however at no time did the lordosis to mount (L/M) ratios (number of lordosis responses by the female divided by the number of mounts by the male) of rats given 25 μg or higher doses of progesterone decline. In the second experiment EB also acted dose-dependently to decrease the lordosis-inhibiting effects of restraint stress. Ovx rats were hormonally primed with various doses of EB followed 48 hr later with 250 μg P. An additional group received 50 μg EB and oil. The lordosis behavior of restrained rats given 0.5, 1.0, or 2.5 μg EB was reduced 5 min after restraint. This reduction in lordosis behavior lasted for 5 min, but by 10 min, L/M ratios had increased. In the third experiment, physiological levels of hormones protected against a stress-induced decline in lordosis

behavior. Proestrous rats showed no decline in lordosis after 5 to 60 min of restraint. In the final experiment, after intraperitoneal treatment with ketanserin tartrate (ketanserin), 5 min of restraint reduced lordosis behavior of proestrous rats. Every rat given 1.0 mg/kg ketanserin and restrained for 5 min showed a decline in lordosis behavior by 5 min after restraint. With 0.50 or 0.75 mg/kg ketanserin, L/M ratios of proestrous rats were not reduced by 5 min of restraint. In conclusion these data are consistent with the suggestions that estrogen and progesterone contribute to facilitation of lordosis behavior and that 5-$HT_{2A/2C}$ receptors may attenuate the disruptive effects of stress.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The lordosis reflex (behavior) is a supraspinal reflex exhibited by the female rat in response to mounting by the male rat. Mounting by the male results in stimulation of cutaneous mechanoreceptors in the flanks, posterior rump, and perineum of the female thereby activating the ascending pathway [41]. Ascending information travels through the medullary reticular formation and the lateral vestibular nucleus ultimately terminating in the brainstem [41]. Descending information travels through the lateral vestibulospinal and lateral reticulospinal tracts of the spinal cord [41]. Both the ascending and descending pathways make axonal projections to the midbrain central gray (an important site of integration). The midbrain central gray also receives hypothalamic inputs [41]. Of special importance to the lordosis reflex is input from the ventromedial nucleus of the hypothalamus (VMN). Estrogen increases activation of VMN neurons, some of which innervate the midbrain central gray. Such estrogen-dependent activation is thought to lead to facilitation of lordosis behavior [41]. In the intact naturally cycling female rat, both estrogen and progesterone induce sexual receptivity [7, 61]. In ovariectomized (ovx) rats a period of 16-18 hr of estrogen priming, followed by progesterone treatment, is required to elicit the entire repertoire of sexual behavior exhibited by an intact sexually receptive female [48]. In ovx rats, progesterone treatment is not required for execution of lordosis if the dose of estrogen is sufficiently large [40, 62]. Yet, progesterone facilitates lordosis behavior of estrogen-primed rats and reduces the dose of estrogen priming

required to cause the reflex [5, 39]. Since lesioning of the VMN significantly reduces lordosis behavior, the VMN is assumed to be a critical component in the lordosis reflex circuitry. The VMN is also a site where gonadal hormones act to facilitate lordosis [48, 67]. In ovx rats, localized application of estrogen and progesterone within the VMN is sufficient to elicit the behavior [67]. It has also been reported that estrogen treatment in the VMN increases neuronal activity in the VMN [22]. Exactly how estrogen and progesterone modulate female rat lordosis is not known.

The estrogen receptor-α (ERα) appears to be critical for induction of lordosis. ERα knockout mice fail to show lordosis, while estrogen receptor-β (ER-β) knockout mice do show lordosis behavior when mounted by male mice [36, 38, 43]. Estrogen is presumed to bind to cytosolic intracellular estrogen receptors (ERα) forming an estrogen-receptor complex [7]. In target cells, the estrogen-receptor complex is translocated from the cytosol to the nucleus where it binds to an estrogen response element altering the synthesis of various proteins [7]. Although not all products of the genomic events are known, progesterone receptors are examples of gene products whose activity is induced by estrogen [32, 46, 63].

Following estrogen priming, progesterone accentuates lordosis behavior presumably via binding to the intracellular progesterone receptor [7]. Progesterone enters the target cell and binds to progestin receptors forming a progesterone-receptor complex [7]. The progesterone-receptor complex is translocated from the cytosol to the nucleus where it alters the synthesis of mRNA and proteins [7]. Substantial evidence exists to support the role of the progesterone receptor in progesterone's enhancement of lordosis

behavior. Lordosis is inhibited in estrogen primed ovx females following VMN infusion with antisense oligonucleotides to the progesterone receptor [28, 37].

Also, several neurotransmitters, including serotonin, (5-hydroxytryptamine, 5-HT) are involved in modulation of female sexual behavior [30, 35]. There are at least seven different families of 5-HT receptors [10]. Since Meyerson et al. [35] suggested that 5-HT inhibited sexual receptivity, there has been evidence that 5-HT exerts a tonic inhibition of lordosis behavior [33]. Generally, an increase in 5-HT is associated with inhibition of lordosis behavior [3, 24, 25], while treatments which decrease 5-HT activity increase lordosis behavior [25, 26, 27]. It is now hypothesized that 5-HT may play a dual role in modulating lordosis behavior. Depending on which receptor is activated, 5-HT can either inhibit or facilitate lordosis [21, 33]. Agonists active at 5-HT_{1A} receptors inhibit [1, 2, 16, 20, 51, 54, 56, 58] lordosis behavior, while agonist activation of $5\text{-HT}_{2A/2C}$ receptors increases the behavior [20, 22, 59, 65, 66].

Substantial evidence exists about how the functioning of these receptors ($5\text{-HT}_{2A/2C}$ and 5-HT_{1A}) affects lordosis behavior; less information is available on the necessity for having two opposing 5-HT receptors mediate lordosis. Uphouse [52] suggested that these two receptors might be required to maintain the female's sexual behavior in spite of a mild stressful environment. The social interaction of mating with the male is likely to produce mild stress; yet female sexual behavior still occurs. Investigators have reported increases in 5-HT in various brain regions following stress [6, 47]. Since 5-HT's affinity for 5-HT_{1A} receptors is higher than its affinity for $5\text{-HT}_{2A/2C}$ receptors, a stress-induced increase in 5-HT may preferentially activate 5-HT_{1A} receptors

thereby inhibiting lordosis behavior [52]. However, simultaneous activation of 5-$HT_{2A/2C}$ receptors may protect against the lordosis-inhibiting effects of stress. It has been reported that following activation of protein kinase C (PKC) by phorbol ester administration, 5-HT_{1A} receptor desensitization occurred within 5 min [42]. While 5-$HT_{2A/2C}$ receptors are positively coupled to phospholipase C and increase PKC [8], 5-HT_{1A} receptors can be negatively coupled to adenylyl cyclase and decrease cyclic adenosine monophosphate (cAMP) [8]. Thus if PKC desensitizes 5-HT_{1A} receptors and if densensitization of 5-HT_{1A} receptors reduces 5-HT_{1A} receptor coupling, then a 5-$HT_{2A/2C}$ receptor-induced activation of PKC may reduce 5-HT's inhibitory impact on lordosis by decreasing the availability of coupled 5-HT_{1A} receptors. Thereby, simultaneous activation of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors may serve to reduce deleterious effects of stress and allow mating to occur.

Estrogen and progesterone have been reported to modify the serotonergic system [4, 45, 53, 55] and to reduce the effects of a 5-HT_{1A} receptor agonist on lordosis behavior [18, 19, 23, 50, 60]. Progesterone was recently shown to attenuate the inhibitory effects of mild restraint stress on lordosis behavior [51]. The present experiments were designed to further explore the potential hormonal suppression of a stress-induced reduction in reproductive behavior. Experiments were further designed to determine if 5-$HT_{2A/2C}$ receptors are involved in attenuation of the effects of stress. The following specific hypotheses were addressed:

1. Progesterone dose-dependently reduces the inhibitory effects of restraint stress on lordosis behavior.

2. Estrogen dose-dependently reduces the inhibitory effects of restraint stress on lordosis behavior.

3. Proestrous rats are protected against the inhibitory effects of restraint (immobilization) stress.

4. The 5-$HT_{2A/2C}$ receptor protects against the effects of restraint stress on lordosis behavior.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

Disposable mouse decapicone restrainers were purchased from Braintree Scientific Inc. (Braintree, MA). Progesterone, ketanserin tartrate (ketanserin) (3-[2-[4-(4-fluorbenzoyl)-1-piperdinyl]ethyl]-2,4(1H,3H)-quinazolinedione), estradiol benzoate, and sesame seed oil were purchased from Sigma Chemical Co. (St. Louis, MO). Isoflurane (AErrane®) was purchased from Baxter Pharmaceutical Products Inc. (Deerfield, IL) and methoxyflurane (Metofane®) was obtained from Pitman Moore (Mundelein, IL). Suture materials were purchased from Butler Co. (Arlington, TX). All other supplies were purchased from Fisher Scientific (Houston, TX).

B. GENERAL METHODS

Animal and housing conditions:

Female rats (CDF-344) were purchased as adults or bred in the TWU animal facility from stock obtained from Sasco Laboratories (Wilmington, MA). TWU bred rats were weaned at 25 days of age. Purchased and TWU rats were housed two or three per cage in polycarbonate shoebox cages with food (rat chow) and water available ad lib. The animal facility rooms were maintained at 25°C and 55% humidity, with lights on from 12 midnight to 12 noon.

Vaginal smears:

Beginning at 60 days of age females were monitored daily for vaginal cyclicity for at least two complete estrous cycles. The female's vagina was flushed with deionized water containing methylene blue and the cell types present were determined by viewing under the light microscope. Smears with clusters of nucleated and/or cornified epithelial cells but an absence of leucocytes were judged as proestrous smears; the presence of predominantly cornified cells, a day after a proestrous smear, represented an estrous smear. Leucocytes present alone or with some nucleated and/or cornified cells indicated a diestrous smear.

Ovariectomies:

Female rats, approximately 60-90 days old, were anaesthetized with Metofane® or AErrane® and a single incision was made across the abdomen. The abdominal muscle was opened and the ovarian tissue found, ligated, and removed. The muscle incision was sutured and the epidermal layer was stapled with a tissue stapler.

Hormone and drug administration:

Hormonal priming began 2-4 weeks after ovariectomy and all hormone injections were administered between 9 and 10 AM. Hormones were dissolved in sesame seed oil and injections were given subcutaneously (SC) in a volume of 0.1 ml per rat. Ketanserin was dissolved in deionized water and administered intraperitoneally (IP) at a volume of 1.0 ml/kilogram rat.

Evaluation of sexual behavior:

At least one hr prior to lights-off on the day of testing, rats were moved in their home cage to the testing room where the males are housed. Testing for female sexual behavior was initiated within 1-3 hr after colony lights-off and 4-6 hr after the progesterone or oil injection. Visibility was aided by a red light. Females were placed in the home cage of a sexually experienced stud male. Sexual receptivity was quantified as the lordosis to mount (L/M) ratio (number of lordosis responses by the female divided by the number of mounts by the male). Lordosis quality was measured as previously described by Hardy and DeBold [15]. A lordosis failure was given a score of 0.0 and a lordosis response with minimal arching of the back was given a score of 1.0. An intermediate reflex was scored as 2.0, a normal reflex scored as 3.0; and an exaggerated reflex was scored as 4.0. Although the L/M ratio and the lordosis quality both are indicators of sexual activity, they provide different information about female sexual behavior. The L/M ratio demonstrates the frequency with which the female shows a lordosis response when mounted by the male. The lordosis quality is a subjective index of the degree of back arching that the female shows when mounted by the male.

C. SPECIFIC METHODS

Experiment 1: Effects of progesterone modulation on lordosis behavior after restraint stress.

Rats approximately 60-90 days old were ovx. Two weeks later, rats were injected with 10 μg of estradiol benzoate followed 48 hr later with 2.5, 5.0, 10, 25, 125, 250, or 500 μg progesterone, or 10 μg estradiol benzoate followed by the vehicle (sesame seed

oil). Injections were given SC in a volume of 0.1 ml/rat. After a 10 mount pretest, experimental rats were immobilized for 5 min and control rats were returned to their home cage for 5 min. Immediately after restraint or the home cage experience, females were put back into the male's cage and sexual behavior was monitored for 3 consecutive 5 min intervals. Females were then returned to the home cage for 15 min and were retested for 10 mounts. The mean ± S.E. L/M ratios and lordosis quality were compared. Data were evaluated by repeated measures ANOVA with time after experience as the repeated factor. An alpha level of 0.05 was required to reject the null hypothesis [68]. Differences in the proportion of rats inhibited post-restraint were compared with chi-square procedures [68].

Experiment 2: Effects of estrogen modulation on lordosis behavior after restraint stress.

Procedures were the same as for experiment 1, but rats were injected SC with varying doses of estradiol benzoate, followed 48 hr later with 250 μg progesterone. Additional rats were injected with 50 μg estradiol benzoate followed by the vehicle (sesame seed oil). After a 10 mount pretest, experimental rats were then immobilized for 5 min and control rats were returned to their home cage for 5 min. Sexual behavior was tested after restraint as described in experiment 1. The mean ± S.E. L/M ratios and lordosis quality were compared. Data were evaluated by repeated measures ANOVA with time after experience as the repeated factor. An alpha level of 0.05 was required to reject the null hypothesis [68]. Differences in the proportion of rats inhibited post-restraint were compared with chi-square procedures [68].

Experiment 3: Effects of restraint stress on lordosis behavior of the proestrous rat.

Regularly cycling proestrous female rats, approximately 60-90 days old, were pretested for sexual behavior in the home cage of a sexually experienced stud male, one-two hr after lights-out at 12 noon. A pretest of 10 mounts was done to assure sexual receptivity prior to restraint. Females were then restrained (immobilized) for 5, 15, 30, or 60 min, or were returned to the home cage for an equivalent length of time. The mean ± S.E. L/M ratios and lordosis quality were compared. Data were evaluated by repeated measures ANOVA with time after experience as the repeated factor. An alpha level of 0.05 was required to reject the null hypothesis [68].

Experiment 4: Effect of a 5-$HT_{2A/2C}$ receptor antagonist on lordosis behavior after restraint.

Procedures were the same as for experiment 3. After a 10 mount pretest, proestrous rats were injected IP with 0.50, 0.75, or 1.0 mg/kg of the 5-$HT_{2A/2C}$ receptor antagonist, ketanserin, or with an equivalent volume of deionized water. Experimental rats were restrained for 5 min and control rats were then returned to their home cage for 5 min. Sexual behavior was tested after restraint as described for experiment 1. The mean ± S.E. L/M ratios were compared. Data were evaluated by repeated measures ANOVA with dose and type of experience as main factors for determination of the effects of restraint [68].

CHAPTER III

RESULTS

Experiment 1: Effects of progesterone modulation on lordosis behavior after restraint stress.

The first experiment was designed to determine if progesterone dose-dependently reduced the inhibitory effects of restraint stress on lordosis behavior. Ovariectomized rats were hormonally primed with 10 μg estradiol benzoate followed 48 hr later with the vehicle (sesame seed oil) or with 2.5, 5.0, 10, 25, 125, 250, or 500 μg progesterone. One hundred receptive rats were included in the study.

Progesterone dose-dependently reduced the lordosis-inhibiting effects of restraint stress on lordosis ($F_{6, 93} = 16.37$, $p \leq .0001$) (Figure 1). Since the decrease in the L/M ratio after restraint was transient, there was also a significant interaction between time and treatment ($F_{24, 372} = 20.18$, $p \leq .0001$). Rats that were given oil or 2.5, 5.0 or 10 μg progesterone and restrained for 5 min showed a significant decrease in their L/M ratio by 5 min after restraint (Dunnett's $q_{372, 7} \geq 2.57$, $p \leq .05$) while no such decrease was apparent for rats given 25 μg or more of progesterone (Figure 1). In addition at 5 min after restraint every progesterone treatment group was significantly different from rats given estrogen and oil (Tukey's $q_{327, 7} \geq 4.17$, $p \leq .05$) (Figure 2). As the dose of progesterone increased from 0 to 25 μg, there was a significant increase in the L/M ratio at 5 min after restraint ($F_{1, 47} = 35.82$, $p \leq .0001$) (Figure 2). From 0 to 5 μg

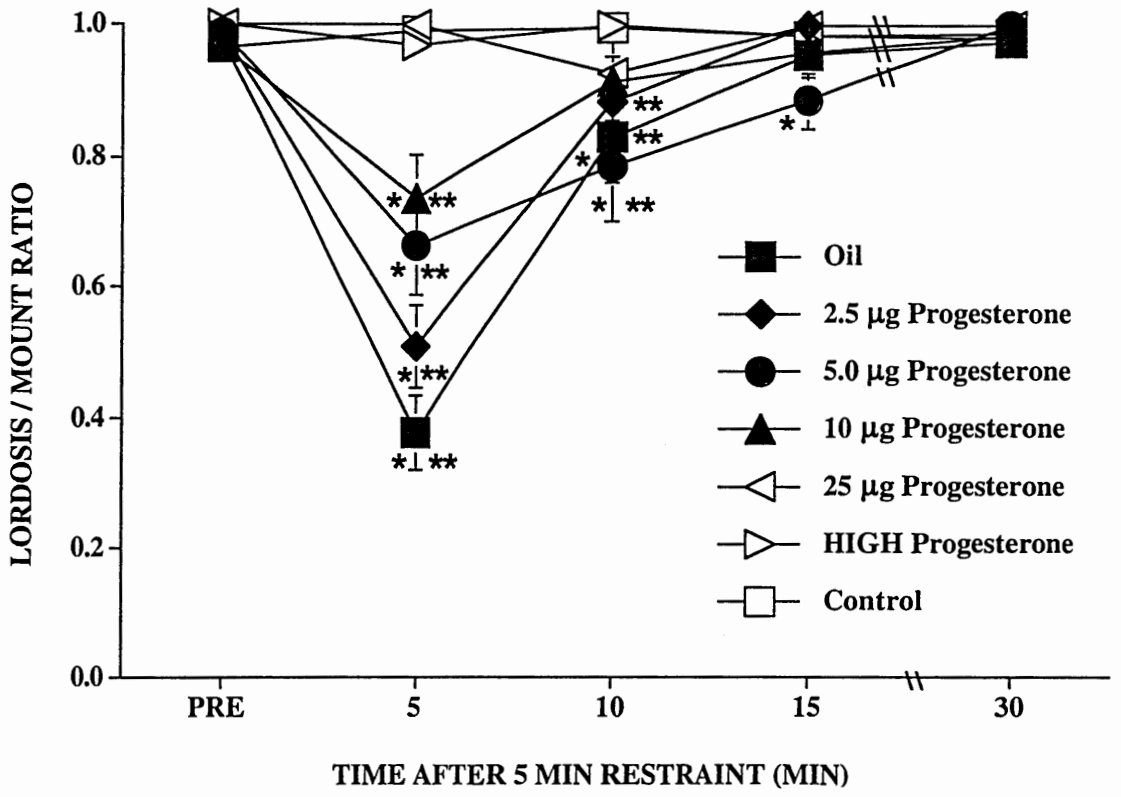

Figure 1. Progesterone dose-dependently modulates the lordosis-inhibiting effects of restraint stress.

Ovariectomized rats were hormonally primed with 10 µg estradiol benzoate followed by oil or with 2.5, 5.0, 10, 25 µg, or higher (HIGH i.e. 125, 250, or 500 µg) doses of progesterone. Four to six hr after the progesterone or oil injection, rats were tested for sexual receptivity (PRE). Rats with L/M ratios of 0.7 or greater were considered sexually receptive and were included in the study with restraint stress. Rats were then restrained for 5 min or returned to the home cage for 5 min. Rats that were given the higher doses of progesterone and restrained for 5 min have been pooled for presentation. Rats that were returned to the home cage have also been pooled for presentation. Immediately after restraint or the home cage experience (control), females were put back onto the male's cage and sexual behavior was monitored for 3 consecutive 5 min intervals. Females were then returned to the home cage and retested for 10 mounts 15 min later. Data are the mean ± S.E. of the L/M ratios for each test interval. N's respectively were 8, 8, 10, 15, 7, 20, and 32. Single asterisks indicate significant differences, within injection groups, from the appropriate pretest interval. Double asterisks indicate intervals where restrained hormone primed rats differed significantly from the hormone primed home cage group.

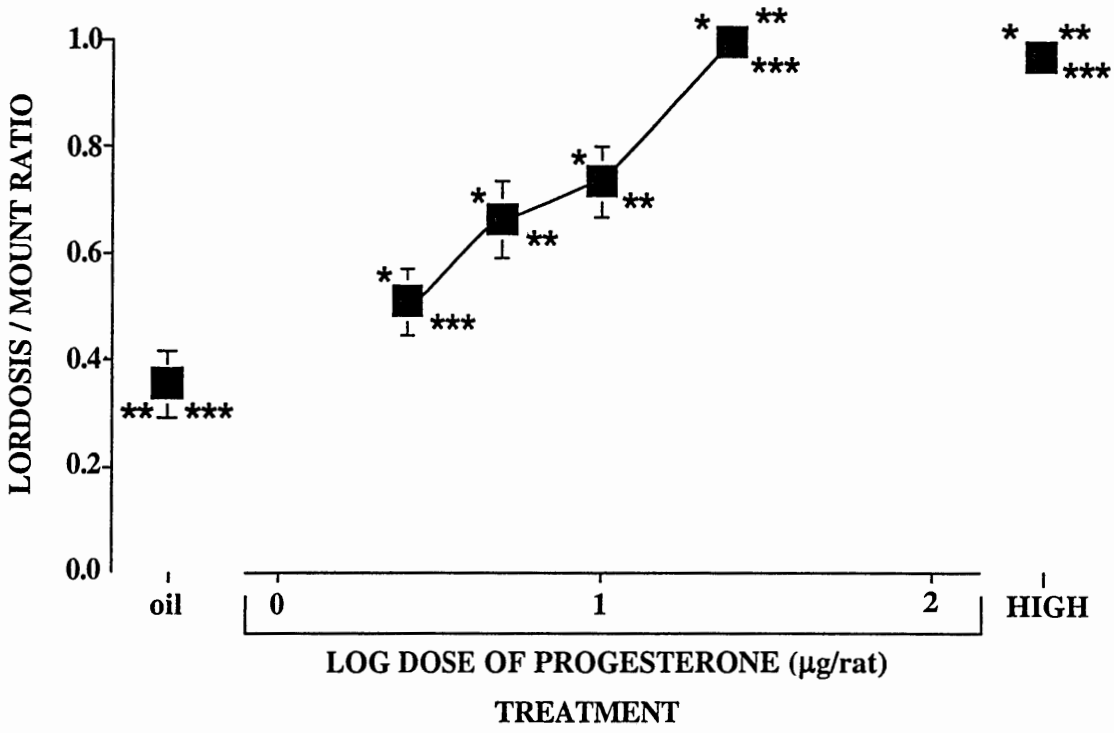

Figure 2. Dose-dependent effects of progesterone on lordosis behavior 5 min after restraint stress.

Data are the mean ± S.E. of the L/M ratios for the first 5 min test interval after the restraint experience. Data are for rats given oil or 2.5, 5.0, 10, or 25 µg or higher (HIGH i.e. 125, 250, or 500 µg) doses of progesterone and are for the same animals shown in Figure 1. Single asterisks indicate a significant difference from rats injected with estradiol benzoate and oil. Double asterisks indicate a significant difference from rats given estradiol benzoate and 2.5 µg progesterone. Triple asterisks indicate significant differences from rats given estradiol benzoate and 5.0 µg progesterone.

progesterone, each increase in dose led to a significant increase in the L/M ratio (Tukey's $q_{327,7} \geq 4.17$, $p \leq .05$). Five and 10 μg progesterone, however, were not significantly different from each other, but were both significantly different from rats given 25 μg or higher doses of progesterone (Tukey's $q_{327,7} \geq 4.17$, $p \leq .05$). At 5 min after restraint, rats in the 25 μg or higher dose of progesterone or home cage treatment groups were not significantly different (Tukey's $q_{327,7} \geq 4.17$, $p \leq .05$) (Figure 2).

By 10 min after restraint L/M ratios had increased but were still significantly lower than the pretest for rats given 0 or 5 μg (Dunnett's $q_{372,7} \geq 2.57$, $p \leq .05$) (Figure 1). Rats given 5.0 μg progesterone were the only group still showing a significant reduction in the L/M ratio 15 min after restraint (Dunnett's $q_{372,7} \geq 2.57$, $p \leq .05$) (Figure 1). At no time during the testing period did rats in the home cage condition (control) or rats given 25 μg or higher (125, 250, or 500 μg) doses of progesterone show a significant decline in the L/M ratio (Dunnett's $q_{372,7} \geq 2.57$, $p \leq .05$) (Figure 1). Not only was there a dose-dependent effect of progesterone on the decrease in L/M ratios after restraint, when lordosis inhibition at 5 min was defined as a L/M ratio less than 0.7, there was a dose-dependent effect of progesterone on the proportion of rats inhibited by the restraint ($\chi^2 = 31.26$, $p \leq .0001$) (Table1). From 0 to 25 μg progesterone, there was a significant linear component to this dose-dependency ($F_{1,4} = 48.98$, $p \leq .006$) and there was a significant correlation ($r = 0.971$, $p < .006$) between dose of progesterone and the percentage of rats that were inhibited. There was also a modest, but statistically

Hormone Priming	Number Inhibited After Restraint	Number Not Inhibited After Restraint	Percent Inhibited After Restraint
10 μg EB + oil	7	1	88
10 μg EB + 2.5 μg P	6	2	75
10 μg EB + 5.0 μg P	5	5	50
10 μg EB + 10 μg P	7	8	47
10 μg EB + 25 μg P	0	7	0
10 μg EB + HIGH	0	20	0

Table 1. Effects of dose of progesterone on the proportion of rats showing a decrease in L/M ratios 5 min after restraint.

Data are the number of rats inhibited after restraint, number of rats not inhibited by restraint and the percent of rats showing an inhibition of lordosis ratios 5 min after restraint. Inhibition was defined as an L/M less than 0.7. Data are for rats given 2.5, 5.0, 10, 25 μg, or higher (HIGH i.e. 125, 250, or 500 μg) doses of progesterone (P) and are from the same animals shown in Figure 1. All rats received 10 μg estradiol benzoate (EB) 48 hr before progesterone or oil.

significant, decline in lordosis quality (data not shown) after restraint ($F_{6, 93} = 14.49$, $p \leq .0001$).

Experiment 2: Effects of estrogen modulation on lordosis behavior after restraint stress.

The second experiment was designed to determine if estrogen dose-dependently reduced the lordosis-inhibiting effects of restraint stress. Ovariectomized rats were hormonally primed with 0.5, 1.0, 2.5, 4.0, or 10 μg estradiol benzoate followed 48 hr later with 250 μg progesterone. An additional group received 50 μg estradiol benzoate plus the vehicle. Seventy-one receptive rats were included in the study.

There was a dose-dependent effect of hormone treatment on lordosis behavior after restraint stress ($F_{5, 55} = 14.51$, $p \leq .0001$) and the interaction between time and treatment was also significant ($F_{20, 220} = 11.39$, $p \leq .0001$) (Figure 3). At 5 min after restraint, rats given 0.5, 1.0, or 2.5 μg estradiol benzoate plus progesterone were significantly different from their pretest interval (Dunnett's $q_{220, 6} \geq 2.51$, $p \leq .05$) (Figure 3). Alternatively, rats given 4.0 μg or 10 μg estradiol benzoate plus progesterone or given 50 μg estradiol benzoate only were not significantly different from their pretest interval or each other at 5 min after restraint (Tukey's $q_{220, 6} \geq, 4.03$, $p \leq .05$) (Figure 3). Home cage controls never showed a decrease in the L/M ratio.

Excluding the estradiol benzoate only group, as the dose of estradiol benzoate increased from 0.5 to 10 μg, there was a significant increase in the L/M ratio 5 min after restraint ($F_{1, 36} = 27.97$, $p \leq .0001$). Rats given 0.5, 1.0, or 2.5 μg estradiol benzoate plus progesterone were each significantly different from each other (Tukey's $q_{220, 6} \geq, 4.03$, $p \leq .05$) (Figure 4); additionally all three groups were significantly different from rats

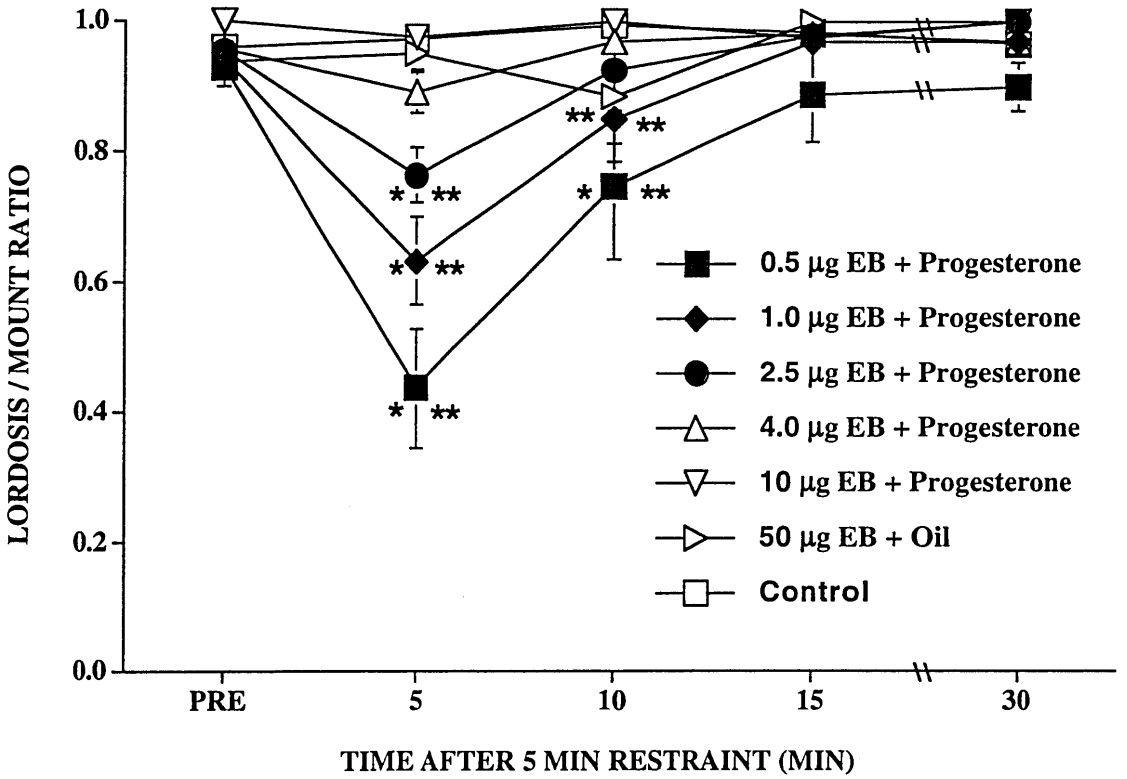

Figure 3. Estradiol benzoate dose-dependently modulates the lordosis-inhibiting effects of restraint.

Ovariectomized rats were hormonally primed with 0.5, 1.0, 2.5, 4.0 or 10 µg estradiol benzoate (EB) plus 250 µg progesterone. Additional rats were given 50 µg estradiol benzoate plus oil. Four to six hr after the progesterone or oil injection, rats were tested for sexual receptivity (PRE). Rats with L/M ratios 0.7 or greater in the pretest were considered sexually receptive and were included in the study. Rats were then restrained for 5 min or returned to the home cage (control) for 5 min. Rats that were returned to the home cage have been pooled for presentation. Immediately after restraint or the home cage experience, females were put back into the male's cage and sexual behavior was monitored for 3 consecutive 5 min intervals. Females were then returned to the home cage and retested for 10 mounts 15 min later. Data are the mean ± S.E. of the L/M ratios at each test interval. N's respectively are 7, 7, 9, 7, 7, and 5 for restrained rats given 0.5, 1.0, 2.5, 4 or 10 µg estradiol benzoate and progesterone or 50 µg estradiol benzoate and oil. Controls include 29 rats. Single asterisks indicate significant differences, within injection groups, from the appropriate pretest interval. Double asterisks indicate intervals where restrained hormonally primed rats differed significantly from the hormone primed home cage group.

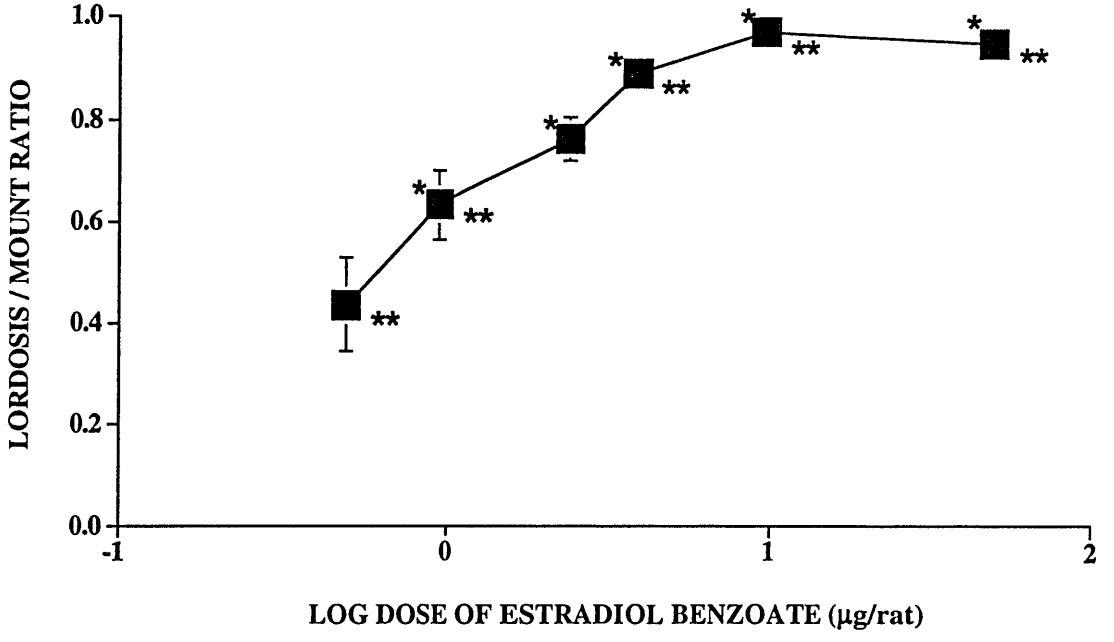

Figure 4. Dose-dependent effects of estradiol benzoate on lordosis behavior 5 min after restraint stress.

Data are the mean ± S.E. of the L/M ratios 5 min after restraint. Data are for rats given 0.5, 1.0, 2.5, 4.0, or 10 µg estradiol benzoate plus progesterone or 50 µg estradiol benzoate and oil and are from the same animals shown in Figure 3. Single asterisks indicate a significant difference from restrained rats given 0.5 µg estradiol benzoate plus progesterone. Double asterisks indicate a significant difference from restrained rats given 2.5 µg estradiol benzoate plus progesterone.

given 4.0 μg or 10 μg estradiol benzoate plus progesterone, rats given 50 μg estradiol benzoate only or rats that remained in the home cage (Tukey's $q_{220,6} \geq 4.03$, $p \leq .05$) (Figure 3).

L/M ratios increased by 10 min after restraint. Although, the L/M ratios of rats given 0.5 or 1.0 μg estradiol benzoate plus progesterone were significantly different from the home cage 10 min after restraint (Dunnett's $q_{220,6} \geq 2.51$, $p \leq .05$), this probably reflects their lower L/M ratios at the beginning of the experiment (Figure 3). Rats given 0.5 μg estradiol benzoate were the only group still significantly different from their pretest interval 10 min after restraint (Tukey's $q_{220,6} \geq 4.03$, $p \leq .05$) (Figure 3).

When rats were injected with 50 μg estradiol benzoate only, there was not a decrease in L/M ratios 5 min after restraint stress, but there was a slight, but significant, decrease 10 min after the restraint (Dunnett's $q_{220,6} \geq 2.51$, $p \leq .05$) (Figure 3). At no time during the testing period did rats given 4.0 μg or 10 μg estradiol benzoate plus progesterone or that remained in the home cage (control) show a significant decline in L/M ratios (Dunnett's $q_{220,6} \geq 2.57$, $p \leq .05$) (Figure 3).

When inhibition of lordosis was defined as a L/M ratio less than 0.7, there was a dose-dependent effect of estradiol benzoate treatment on the proportion of rats inhibited 5 min after restraint ($\chi^2 = 22.37$, $p \leq .05$) (Table 2). There was a significant linear component to this dose-dependency over the range of 0.5 to 4.0 μg estradiol benzoate plus 250 μg progesterone ($F_{1,3} = 175.23$, $p \leq .006$) and there was a significant correlation ($r = 0.994$, $p \leq .006$) between dose of estradiol benzoate and the percentage of rats that were inhibited. There was a modest, but statistically significant, decline in lordosis

Hormone Priming	Number Inhibited After Restraint	Number Not Inhibited After Restraint	Percent Inhibited After Restraint
0.5 μg EB + 250 μg P	6	1	86
1.0 μg EB + 250 μg P	5	2	71
2.5 μg EB + 250 μg P	4	5	44
4.0 μg EB + 250 μg P	0	7	0
10 μg EB + 250 μg P	0	7	0
50 μg EB + oil	0	7	0

Table 2. Effects of the dose of estradiol benzoate on the proportion of rats showing a decrease in L/M ratios 5 min after restraint.

Data are the percent of rats showing inhibition of lordosis ratios 5 min after restraint. Inhibition was defined as an L/M ratio less than 0.7. Data are for rats given 0.5, 1.0, 2.5, 4.0 or 10 μg estradiol benzoate (EB) plus progesterone (P) or 50 μg estradiol benzoate only and are from the same animals shown in Figure 4.

quality (data not shown) after restraint ($F_{6, 63} = 13.57$, $p \leq .0001$). For analysis of lordosis quality one rat was excluded from the 0.5 μg estradiol benzoate plus progesterone restraint treatment group because the rat had an L/M ratio of zero.

Experiment 3: Effects of restraint stress on lordosis behavior of the proestrous rat.

Since gonadal hormones (progesterone and estrogen) were shown to protect against the lordosis-inhibiting effects of restraint in the prior studies, the next experiment was designed to test the hypothesis that physiological levels of hormones protect against the inhibitory effects of restraint stress. Intact regularly cycling female rats were selected on the morning of proestrous.. Thirty-seven rats were restrained for 5, 15, 30 or 60 min, or were returned to the home cage for an equivalent length of time. The L/M ratios before and after the experience are shown in Figure 5. There was no effect of any restraint duration on lordosis behavior of proestrous rats ($F_{4, 32} = .43$, $p > .05$).
Due to a lordosis/mount ratio of zero, one rat was excluded from both the 15 min restraint and home cage treatment groups to assess lordosis quality. There was no effect of any restraint duration on lordosis quality (data not shown) of proestrous rats ($F_{4, 30} = 1.71$, $p > .05$).

Experiment 4: Effect of a 5-$HT_{2A/2C}$ receptor antagonist on lordosis behavior after restraint.

The fourth experiment was designed to test the hypothesis that 5-$HT_{2A/2C}$ receptor activation protects against the effects of restraint stress on lordosis behavior. Intact, regularly cycling female rats were selected on the morning of proestrous. That afternoon rats were pretested for sexual receptivity and then injected intraperitoneally (IP) with

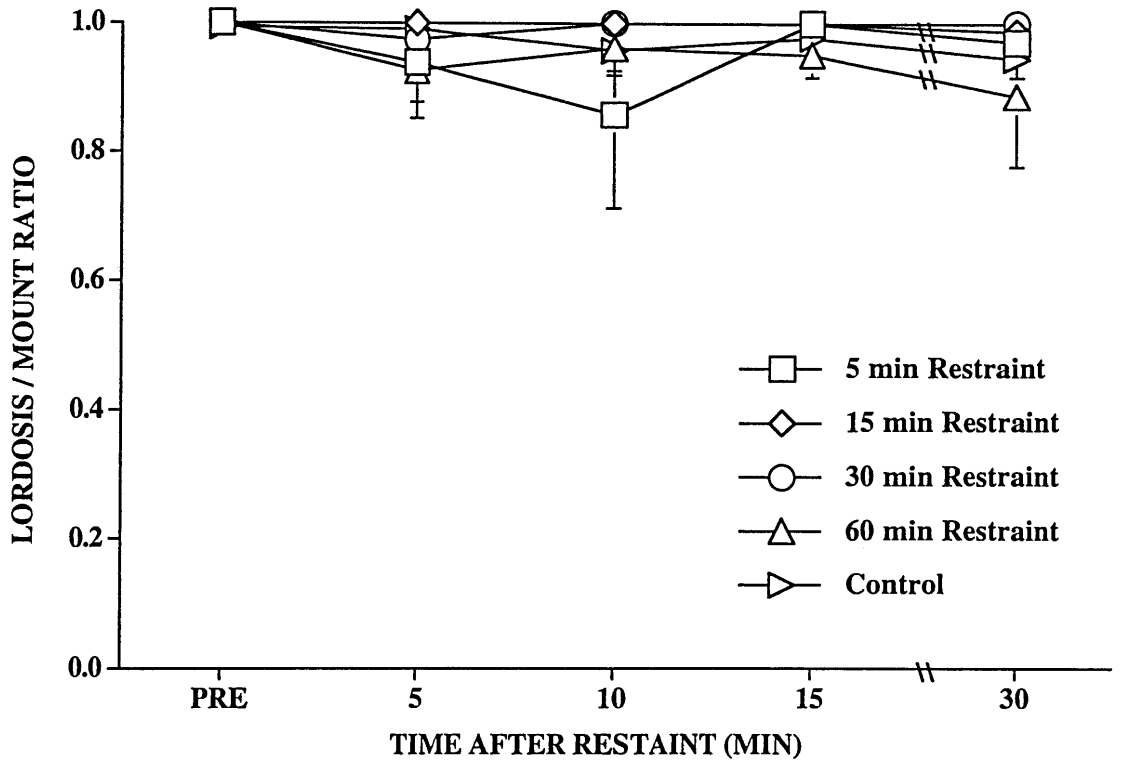

Figure 5. Lordosis behavior of proestrous rats after restraint stress.

Proestrous rats were restrained for 5, 15, 30 or 60 min or were returned to the home cage (control) for an equivalent length of time. Rats that were returned to the home cage have been pooled for presentation. N's respectively were 7, 6, 4, 5, and 16. Immediately after restraint or the home cage experience, females were put back into the male's cage and sexual behavior was monitored for 3 consecutive 5 min intervals. Females were then returned to the home cage and retested for 10 mounts 15 min later. Data are the mean ± S.E. L/M ratios for the pretest and each of the test intervals shown.

0.50, 0.75, or 1.0 mg/kg of the 5-$HT_{2A/2C}$ receptor antagonist, ketanserin, or an equivalent volume of deionized water. Thirty-six rats were then restrained for 5 min or returned to the home cage for 5 min. Lordosis behavior was then monitored as described in earlier studies.

In order to compare the effects of ketanserin in the home cage vs. the restraint experience, the water group was excluded. Data were compared with a two-way repeated measures ANOVA (dose of drug x type of experience) (Figure 6). There were significant effects of restraint vs. no restraint (home cage) ($F_{2, 25} = 12.60$, $p \leq .05$) and dose of ketanserin ($F_{1, 25} = 17.45$, $p \leq .05$) on lordosis behavior. There was also a significant effect of time after the experience ($F_{4, 100} = 5.12$, $p \leq .05$), as well as a significant interaction between time and type of experience ($F_{4, 100} = 2.90$, $p \leq .05$) and between time and dose of ketanserin ($F_{8, 100} = 2.39$, $p \leq .05$). Rats injected with ketanserin and returned to their home cage had L/M ratios similar to their pretest (Tukey's$_{100, 6} \geq 4.10$, $p \leq .05$). In contrast when rats were restrained after treatment with ketanserin, there was a decrease in the L/M ratio (Tukey's$_{100, 6}$ $4.10 \geq$, $p \leq .05$). Rats that were given 1.0 mg/kg ketanserin and restrained for 5 min showed a significant decrease in their L/M ratios by 5 min after restraint (Dunnett's$_{100, 6} \geq 2.55$, $p \leq .05$). Every rat given this treatment exhibited a reduction in the L/M ratio. Although the L/M ratio increased by 10 min after restraint, L/M ratios remained significantly lower than the pretest at all time intervals (Dunnett's$_{100, 6} \geq 2.55$, $p \leq .05$). All other treatments were significantly different from

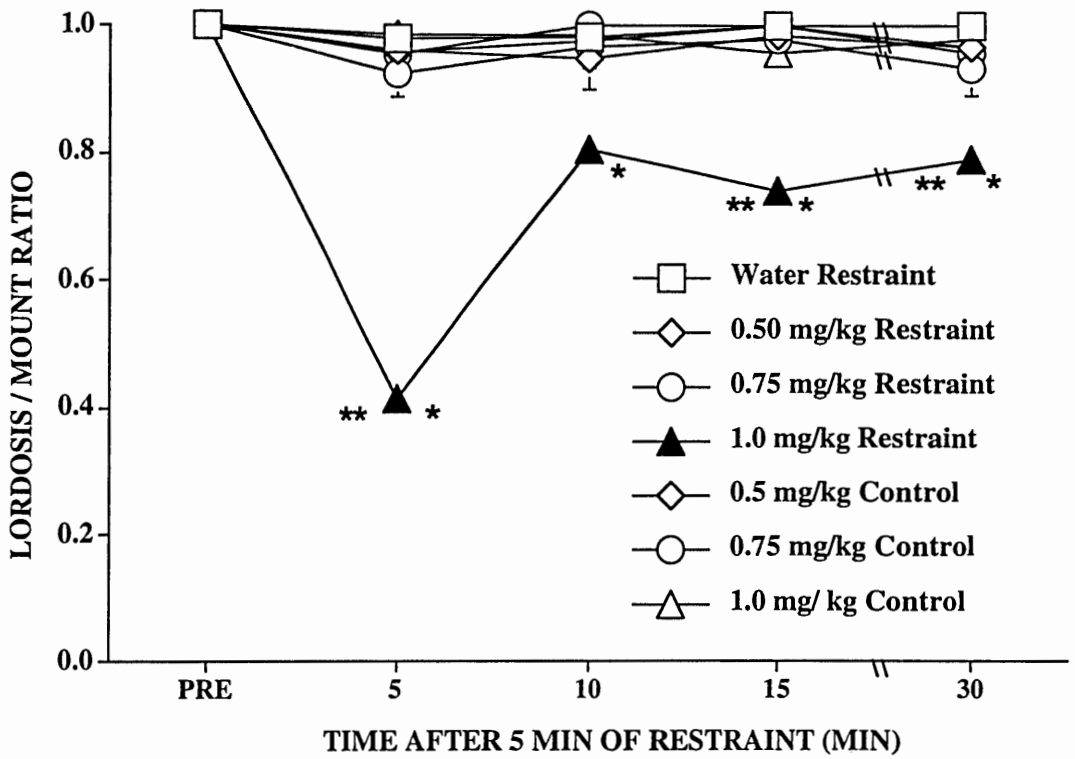

Figure 6. Effects of ketanserin on lordosis behavior of proestrous females after restraint stress.

Proestrous rats were pretested (PRE) for sexual behavior. Immediately after this pretest, rats were injected intraperitoneally (IP) with ketanserin (0.1 ml/100 g body weight) or an equivalent volume of deionized water. Females were either restrained for 5 min or returned to their home cage for 5 min. Immediately after restraint or the home cage experience (control), females were put back into the male's cage and sexual behavior was monitored for 3 consecutive 5 min intervals. Females were then returned to the home cage and retested for 10 mounts 15 min later. The mean ± S.E. of the L/M ratios for rats that were given 0.50, 0.75, or 1.0 mg/kg ketanserin or water (water) and restrained for 5 min are shown. N's respectively were 5, 6, 6, and 5. The mean ± S.E. of the L/M ratios for rats that were given 0.50, 0.75, or 1.0 mg/kg ketanserin and were returned to the home cage are also shown. N's respectively were 3, 5, and 5. Single asterisks indicate significant differences, within injection groups, from the appropriate pretest interval. Double asterisks indicate time points where, within time intervals, rats that were injected with 1.0 mg/kg ketanserin differed from all other groups.

1.0 mg/kg ketanserin plus restraint (Tukey's $s_{100, 6} \geq 4.10$, $p \leq .05$) and for none of these treatments was there a significant decrease in lordosis behavior after restraint (Dunnett's $s_{100, 6} \geq 2.55$, $p \leq .05$).

Due to a lordosis/mount ratio of zero, one rat was excluded from the 1.0 mg/kg ketanserin plus restraint treatment group when lordosis quality was assessed. Data were compared with a two-way, repeated measures ANOVA (dose of drug x experience), with the water group excluded, in order to evaluate the effects of restraint vs. home cage experience. When the effects of ketanserin in the restraint vs. home cage condition were compared, there were no significant effects of type of experience on lordosis quality (data not shown) (all $p > .05$).

Data were compared with a one-way, repeated measures ANOVA, with the home cage group excluded, to evaluate the effects of ketanserin vs. water in the restraint condition (Figure 6). There was a significant effect of the treatment on L/M ratios ($F_{3, 19} = 19.18$, $p \leq .05$). Water plus 5 min of restraint had no effect on the L/M ratios (all $q \leq 2.41$ $p > .05$). However, there was a decline in the L/M ratio when rats were restrained after injection with 1.0 mg/kg ketanserin. This decrease was evident at each time point after restraint (Dunnett's $s_{76, 4} \geq 2.41$, $p \leq .05$). Consequently, there were significant effects of time after experience ($F_{4, 76} = 6.00$, $p \leq .0003$) and the interaction between time and treatment was also significant ($F_{12, 76} = 3.80$, $p \leq .05$).

For analysis of lordosis quality, one rat was excluded from the 1.0 mg/kg ketanserin plus restraint treatment group because the rat had an L/M ratio of zero. Data were compared with a one-way repeated measures ANOVA, with the home cage

excluded, in order to evaluate the effects of ketanserin in the restraint condition. When the effects of ketanserin vs. water were compared in the restraint condition, there were modest, but significant, effects of drug treatment ($F_{3, 18} = 4.02$, $p \leq .05$) and time after experience ($F_{4, 72} = 9.21$, $p \leq .05$) on lordosis quality (data not shown).

CHAPTER IV

DISCUSSION

These studies were designed to evaluate potential hormonal suppression of the effects of stress and to determine if 5-$HT_{2A/2C}$ receptors were involved in attenuation of a stress-induced reduction in female rat lordosis behavior. The role of estrogen and progesterone in sexual receptivity is well established [7, 40, 48]. At low doses of estrogen, females do not exhibit lordosis behavior, but progesterone pushes females beyond a threshold for lordosis to occur. High doses of estrogen, alone, can also facilitate lordosis behavior, but progesterone may still enhance sexual receptivity [48]. Estrogen and progesterone's ability to enhance sexual behavior is thought to occur in part via genomic events consequent to binding to their respective intracellular steroid receptors [7, 28]. Substantial evidence exits to support the role of progesterone and estrogen receptors in female gonadal hormone enhancement of lordosis behavior. For example, VMN infusion with antisense oligonucleotides to the progesterone receptor [28, 37] inhibited progesterone's facilitation of lordosis in ovx estrogen primed rats. Moreover, systemic administration of estrogen receptor antagonists to ovx rats decreased estrogen-induced lordosis [48]. The ERα is currently thought to be required for estrogen's induction of lordosis behavior. Most recently, it was reported that ERα knockout mice do not show sexual receptivity after estrogen priming, while ERβ knockout mice do show lordosis behavior after hormone replacement [36, 38, 43].

Furthermore, it has been demonstrated that estrogen may also enhance lordosis by acting at the progesterone receptor [48].

However, there is also evidence that progesterone may facilitate lordosis by nongenomic mechanisms that are independent of the progesterone receptor [11, 13]. For example, progesterone or one of its metabolites has been reported to enhance GABA $(\gamma\text{-aminobutyric acid})_A$ receptor activation [12], which increases Cl^- conductance and hyperpolarizes neurons, and $GABA_A$ receptor agonists can facilitate lordosis behavior [31]. It is thus unclear if genomic or nongenomic mechanisms account for progesterone's facilitation of lordosis behavior.

Neurotransmitters, including 5-HT, can function to influence the female's position relative to a lordosis threshold. The lordosis-inhibiting effect of 5-HT in the mediobasal hypothalamus results primarily from the neurotransmitter's activation of 5-HT_{1A} receptors [56]. In contrast, agonist activation of $5\text{-HT}_{2A/2C}$ receptors has been associated with facilitation of the behavior [17, 20] and may reduce the inhibitory effects of 5-HT_{1A} receptor agonists [29, 57]. Stress rapidly increases release of 5-HT [6, 47] and increased 5-HT in the mediobasal hypothalamus has been reported to inhibit lordosis behavior [35, 52]. Therefore, stress might be expected to decrease lordosis behavior.

Uphouse [52] recently suggested that the female's susceptibility to treatments that disrupt lordosis behavior may depend on the rat's position relative to a theoretical lordosis threshold. Therefore, mild restraint would be expected to lower sexual receptivity, but only if the female were near the threshold for the behavior to occur. The current data are consistent with this expectation.

Naturally cycling rats showed no decline in lordosis behavior after 5 to 60 min of restraint. However after treatment with the non-selective 5-HT_2 receptor antagonist, ketanserin, sexual behavior was disrupted. Similarly, lordosis behavior of ovx rats primed with 10 μg estradiol benzoate and 500 μg progesterone was not attenuated by stress while, after ketanserin treatment, lordosis was disrupted [62]. In contrast, lordosis behavior of rats primed with only estradiol benzoate was reduced in the absence of ketanserin [51]. These findings allow the suggestion that both 5-HT_2 receptors and progesterone protect against the lordosis-inhibiting effects of restraint stress.

Whether or not these observations about effects of restraint and the 5-HT_2 receptor antagonist reflect similarities in mechanism(s) that may potentially protect against a stress-induced disruption in lordosis behavior is unclear. However, the restraint-induced inhibition of lordosis appears to be dependent on the hormonal state of the female rat. In the current studies, progesterone was shown to dose-dependently prevent the lordosis-inhibiting effects of restraint stress. Several mechanisms might be responsible for this protection, but progesterone's effects against restraint could involve the same mechanisms thought to be responsible for the hormone's facilitation of lordosis behavior. Estradiol benzoate also acted dose-dependently to reduce the lordosis-inhibiting effects of restraint. Like progesterone, estrogen's protective effects against restraint may reside in its ability to enhance mechanisms involved in facilitating lordosis. However, there is also evidence to suggest that estrogen and progesterone's modulation of the 5-HT system could contribute to protection against the negative effects of restraint stress. It is possible that estrogen or progesterone after estrogen priming may decrease

5-HT_{1A} receptor activation. If it is assumed that the lordosis-inhibiting effects of restraint results from increased 5-HT_{1A} receptor activation, then treatments that decrease 5-HT_{1A} receptor activation would be expected to prevent decreases in lordosis behavior. Lakoski [23] reported that estrogen decreased the ability of the 5-HT_{1A} receptor agonist, (±) 8-hydroxy-2-(di-*n*-propylamino)tetralin-HBr (8-OH-DPAT), to suppress 5-HT neuronal firing. Several investigators have also reported that estrogen treatment reduced the potency of 8-OH-DPAT to inhibit lordosis behavior [19, 50, 58]. Additionally, progesterone, following estrogen treatment, has been reported to decrease the ability of a 5-HT_{1A} receptor agonist to inhibit lordosis [51]. Thus, if estrogen and progesterone decrease 5-HT_{1A} receptor activation and if activation of 5-HT_{1A} receptors decreases lordosis behavior, it is not unreasonable that both hormones may protect against the disruptive effects of restraint stress.

Wolf et al. [65] reported that activation of 5-HT_{2C} receptors could facilitate lordosis behavior. Increases in 5-$HT_{2A/2C}$ receptor density have been reported at proestrous and following estrogen treatment [4, 49]. This observation, coupled with the accentuation of restraint by ketanserin, allows the suggestion that an increase in 5-$HT_{2A/2C}$ receptors could be important in estrogen's protective effects. Moreover, progesterone, after estrogen priming has been reported to increase 5-HT_2 receptor function [64]. This is important because Uphouse [57] reported that a 5-HT_2 receptor agonist prevented the inhibitory actions of 5-HT and a 5-HT_{1A} receptor agonist on lordosis behavior. Therefore, upon activation of both receptors, 5-$HT_{2A/2C}$ receptors can attenuate the lordosis-inhibiting effects due to 5-HT_{1A} receptor activation. Thus, in the presence of

enhanced 5-HT_2 receptor function, potential stress-induced increases in 5-HT might be ineffective in decreasing lordosis behavior.

Generally, decreases in 5-HT activity would be expected to facilitate lordosis behavior [52]. It is therefore possible that progesterone's protective effects could reside in it ability to decrease 5-HT release. Farmer et al. [9] reported that progesterone, following estrogen treatment, led to a decline in extracellular 5-HT in the hypothalamus, and Gereau et al. [14] reported that progesterone following estrogen decreased 5-HT in the VMN. Therefore, if restraint attenuates lordosis by increasing extracellular levels of 5-HT and if progesterone can reduce 5-HT's inhibitory impact on lordosis behavior, then progesterone might be expected to protect against a restraint-induced decline in lordosis.

Alternatively, the protective effects of progesterone may involve, in part, putative anxiolytic actions. It has been reported that progesterone or one of its metabolites exerted anxiolytic actions on behavioral indices of anxiety in female rats [44]. The current data would thus be consistent with reports supporting anxiolytic actions of progesterone.

In summary, the present findings demonstrate that progesterone dose-dependently reduced decrements in lordosis behavior following 5 min of restraint stress. Additionally, estradiol benzoate dose-dependently attenuated a restraint-induced decline in lordosis. Thus, both estradiol benzoate and progesterone protected against the inhibitory effects of restraint stress on lordosis behavior. Similar to findings with estrogen and progesterone treated ovx females, 5 to 60 min of restraint stress did not disrupt lordosis behavior of proestrous rats. However, 1.0 mg/kg ketanserin accentuated the inhibitory effects of

restraint stress, so that a decline in the lordosis behavior was seen in proestrous rats. The results from these experiments are consistent with ideas of progesterone as an anxiolytic compound, that the functional impact of restraint stress are diminished as the female moves from the sexually non-receptive state to the sexually receptive state, and that 5-$HT_{2A/2C}$ receptor activation prevents the inhibition associated with restraint stress. Further, studies will be required to determine the precise mechanisms involved.

CHAPTER V

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