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Inhibition of Rat Sexual Behavior by Antisense Oligonucleotides to the Progesterone Receptor*

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ABSTRACT

To test further the idea that sexual behavior in rodents is mediated via the progesterone receptor (PR) in the ventromedial nucleus of the hypothalamus, antisense and sense oligonucleotides to progesterone receptor were administered intracerebroventricularly into the third cerebral ventricle of ovariectomized estrogen-primed animals. Progesterone-facilitated sexual behavior was inhibited in animals treated with antisense oligonucleotides, with proceptive and receptive responses being minimal or completely suppressed. Sexual behavior was not altered by control sense oligonucleotides. *In vitro* binding assays of the cytosol progesterone receptors demonstrated a 52.2% reduction of PRs in the hypothalamus of animals that received antisense oligonucleotides, suggesting a reduction in PR synthesis. These data suggest that a threshold level of estrogen-induced hypothalamic PR is critical in the regulation of progesterone-facilitated sexual behavior in female rats. (*Endocrinology* **135:** 1409–1414, 1994)

THE STEROID hormones estrogen (E) and progesterone (P) regulate cellular functions in the brain that control sexual behavior in the rat (1). Facilitatory effects of P on lordosis are dependent on the prior conditioning of neural tissues with E. The cellular basis for regulatory action of E is believed to involve activation of E receptors (ER) in the ventromedial nucleus (VMN) (2) of the hypothalamus, which, in turn, act as ligand-activated transcription factors, altering the expression of a number of hypothalamic genes, including P receptor (PR) (3–9). E induction causes an increase in PR messenger RNA (mRNA) levels in the VMN of the rat hypothalamus (10), followed by enhanced PR synthesis (11). Furthermore, a strong correlation exists between the E-induced increase in PR-binding sites in the VMN and the expression of female reproductive behavior in rats (11–13). Studies using intracranial hormone implants (14–17) and localized brain lesions (18, 19) suggest that the hypothalamus, including the ventromedial nucleus (VMN), is the key site of action of both E and P for inducing lordosis. Application of inhibitors to RNA and protein synthesis to the VMN blocks E- plus P-induced lordosis (20–23), suggesting an involvement of gene expression in the control of lordosis.

The action of P has been suggested to be mediated by PR, because the P antagonist RU 38486 inhibits P-facilitated lordosis (24). This antagonist is a competitive inhibitor of hypothalamic PR binding both *in vivo* and *in vitro*. However, the role of neural PR in the facilitation of female reproductive behavior requires further clarification, because RU 38486 can competitively bind and reduce the soluble pool of PR by 50%. RU 38486 has potent antiglucocorticoid activity (25), so that the observed inhibition could be due to interference in glucocorticoid physiology. To substantiate that female sexual behavior in rodents is mediated via E-induced genomic activation of hypothalamic PR, we examined the effects of inhibition of PR gene expression in the VMN by intracerebroventricular (icv) administration of antisense oligonucleotides to PR mRNA on proceptive and receptive behaviors in rats.

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Materials and Methods

Behavioral testing of animals

Ovariectomized Sprague-Dawley rats (160–180 g BW) were obtained from the supplier (Sasco or Harlan, Houston, TX). The animals were housed with a 12-h light, 12-h dark cycle and given food and water *ad libitum*. A week after their arrival, the animals were administered hormones and tested for sex behavior. 17β-Estradiol benzoate (EB; 10 μg in sesame oil) was injected sc, followed by P sc (100 μg in sesame oil) 48 h later. Four hours after P administration, the animals were tested for sex behavior with sexually active males housed in a 50 × 45 × 24-cm polystyrene arena. The proceptive and receptive behaviors of each female rat in the presence of the male were observed for 10 min, scored, and recorded. Proceptivity in the female was measured in terms of 1) hopping and darting, 2) ear wiggling, and 3) approaches to the male. Receptive behavior was measured by evaluating 1) the number of lordoses, 2) acceptance of the male by the female, 3) the number of mounts by the male, and 4) lordosis quotient. All animals that exhibited high levels of lordosis were used in the experiments. To minimize ambiguity and provide maximal quantification, the results of the experiments are expressed as the lordosis quotient (LQ), defined as the percentage of the number of complete lordosis responses (perineum elevated, all four legs extended from the initial crouch position, and head at an angle of 45° from the floor) divided by the number of mounts by the male (26).

All of the experiments described below were performed a minimum of four times each, and the observations were recorded manually on video and made in a double blind manner.

Third ventricle cannulation

A stainless steel cannula (23 gauge) was implanted adjacent to the VMN of the hypothalamus into the third ventricle of the female rats using a Lab Standard stereotaxic instrument (Stoelting, Wood Dale, IL). For cannulation, the animals were anesthetized with a combination anesthetic (0.5 ml/kg) containing ketamine (42.8 mg/ml), xylazine (8.6 mg/ml), and acepromazine (1.4 mg/ml), and the rat's head was fixed in the stereotaxic equipment. The coordinates used for the third ventricle were: antero-posterior, bregma −3.3 mm; lateral, just on the midline (above superior longitudinal sinus); and dorsoventral, −8.5 mm. The procedure for cannulation was similar to that described by Antunes-Rodrigues and McCann (27). Surgical tools required for cannulation and microinjections were obtained from the Bioinstrumentation Department (University of Texas Health Science System, Dallas, TX) and are the same as those described by Antunes-Rodrigues and McCann (27). Animals were allowed to recover from surgery for 1 week before use in experiments.

Dose response with P

Cannulated animals were injected sc with EB (10 μg). Forty-eight hours later, P in sesame oil at varying doses (*e.g.* 0.5, 5, 50, and 2 μg) was injected into the third ventricle (icv). The proceptive and receptive behaviors of the animals were observed at various time periods after P administration and recorded.

Inhibition of sexual behavior with P receptor antagonists

Cannulated animals were primed with E (10 μg). One hour before icv administration of P (2 μg), the PR antagonists RU 38486 (2 μg; Roussal-UCLAF, Paris, France) and ZK 98299 (2 μg; Schering, Berlin, Germany) were injected icv into the third ventricle. Proceptive and receptive behaviors of the animals were observed and recorded as described above.

Administration of sense and antisense oligonucleotides

Two sets of 20-mer antisense (PRAs) and sense (PRS) oligonucleotides, one set phosphorothioated and the other not, to rat P receptor A were synthesized [Synthecell (Rockville, MD) and Genosys (Conroe, TX)]. The oligonucleotides were designed such that they included the ATG site in the A form of the P receptor:

Gene sequence:
5′-TG TTG TCC CCG CTC ATG AGC 3′
3′-AC AAC AGG GGC GAG TAC TCG-5′

mRNA:
5′-UG UUG UCC CCG CUC AUG AGC-3′

Sense oligo (PRS):
5′-TG TTG TCC CCG CTC ATG AGC-3′

Antisense oligo (PRAs)
5′-GC TCA TGA GCG GGG ACA ACA-3′

Cannulated female rats that exhibited high levels of proceptive and receptive sexual behaviors in pretests were injected sc with EB (10 μg). At the same time, antisense and sense phosphorothioated oligonucleotides at 4, 1.6, and 0.8 nmol were administered via the third ventricle. The oligonucleotides were administered again 24 h later via the same route; 48 h after E stimulation, P was administered icv, and sexual behavior was observed at 30 min and again at 180 min. The phosphorothioated oligonucleotides were used in these experiments because of their resistance to nuclease degradation and their effectiveness over longer durations (28, 29). Another group of rats received a nonspecific oligonucleotide containing a 20-mer sequence for directional cloning into the SP6 vector (NS-O). These oligonucleotides served as nonspecific controls. Positive controls included cannulated rats that received EB sc, followed by icv injection of P (2 μg) 48 h later and observation of sexual behavior 30 and 180 min after P treatment. Similar experiments were performed with varying doses of the nonphosphorothioated oligonucleotides. In another set of experiments, similar treatments were given, except that a single administration of oligonucleotides was given at the time of E priming (0 h), and the second injection was excluded.

Cytosol P receptor assays

Cytosol P receptors were assayed as described previously (30). All steps were carried out at 0–4 C. The mediobasal hypothalamus was dissected out, bounded rostrally by the caudal edge of the optic chiasm and caudally by the caudal edge of the mamillary bodies. Diagonal cuts were made extending from the lateral hypothalamic fissures to the midpoint of the corpus callosum to form the lateral boundaries, and a cut below the level of the fornix formed the dorsal boundary. Tissues were homogenized in TEGT (10 mM Tris-HCl, 1.5 mM Na_2EDTA, 10% glycerol, and 12 mM monothioglycerol, pH 7.4) using a Polytron tissue grinder with a PT-7 probe (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged at 48,000 × *g* for 30 min, and aliquots of the high speed supernatant were incubated with 0.4 nM [^{3}H]R 5020 (final concentration; SA, 89.1; New England Nuclear Corp., Boston, MA) with or without 100 nM unlabeled P. After a 4-h incubation at 0 C, bound and free [^{3}H]R 5020 were separated by gel filtration on 5 × 60-cm columns of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ). The protein peak was eluted into scintillation vials, scintillation fluid was added, and the samples were counted in a Packard Tri-Carb liquid scintillation spectrophotometer (Packard, Downers Grove, IL). The concentrations of cytosol protein were assayed by the method of Bradford (31), and the data are presented as femtomoles of [^{3}H]R 5020 specifically bound per mg protein.

Results

General behavioral testing

EB- and P-treated (sc) animals exhibited high levels of proceptive (ear wiggling, darting, and hopping) and receptive behaviors. Mounting by the male resulted in the lordosis posture of the female and acceptance of the male by the female (receptivity). Third ventricle cannulations of ovariectomized rats did not decrease their ability to display these behaviors (Table 1).

Dose response to P

Although it has been demonstrated that smaller doses of P were adequate in inducing mating behavior in hamsters when administered directly into the lateral ventricles of the brain, no dose-response studies were performed to determine the minimum dose of P required to induce lordosis (32). As we intended to administer various hormones and oligonucleotides via the third cerebral ventricle, we examined the effects of different doses of P on mating behavior. Intracerebroventricular administration of P (2 μg) in E-primed rats resulted in their display of proceptive behavior beginning at 30–45 min and lasting for up to 3 h. At this dose, more females exhibited proceptive and receptive behaviors, and their average LQs were higher than those of animals that received lower doses (Fig. 1). Lower doses of P were characterized by irregular displays of lordosis, frequent rejection behavior, and low levels of hopping, darting, and ear wiggling, whereas higher doses resulted in reliable and higher levels of proceptive and receptive behaviors. In the absence of P, E-primed animals displayed minimal receptive behavior (lordosis) and no proceptive behavior. Administration of the vehicle alone (in the absence of P) did not induce proceptive and receptive behaviors. Thus, at the higher doses of P (50 ng to 2 μg), all

TABLE 1. Effect of icv administration of P on lordosis response of female rats

Behavior	Progesterone sc	Progesterone icv
Proceptive behavior		
Ear wiggle	32.3 ± 3.1	27.8 ± 2.9
Hop-dart	23.1 ± 2.9	17.8 ± 1.9
Receptive behavior		
Acceptance	23.8 ± 2.3	17.6 ± 1.9
No. of lordoses	23.8 ± 2.3	17.6 ± 1.9
No. of mounts	24.3 ± 2.5	26.3 ± 2.5
LQ	98.0 ± 1.2	98.6 ± 0.9

Proceptive and receptive behavior (as described in *Materials and Methods*) of each female rat in the presence of a male was scored and recorded. The results were expressed as the LQ, defined as a percentage of the number of complete lordosis responses divided by the number of mounts by the male. The responses of each female rat (n = 6) in the presence of a male rat for 10 min was scored individually, and the mean ± SEM were determined. Acceptance, The number of times the female allowed the male to mount. Ovariectomized female rats were primed with EB (10 μg) in 0.1 ml sesame oil, sc, and administered 100 μg P, sc, 48 h later. Four hours after P administration, the animals were tested for sexual behavior in the presence of sexually active males. Stainless steel cannulae were stereotaxically implanted into the third cerebral ventricle of ovariectomized animals. A week after surgery the animals were primed sc with EB (10 μg), and P (2 μg) was microinjected into the third cerebral ventricle (ICV) and their proceptive and receptive behaviors in the presence of males were observed 30 min after P administration.

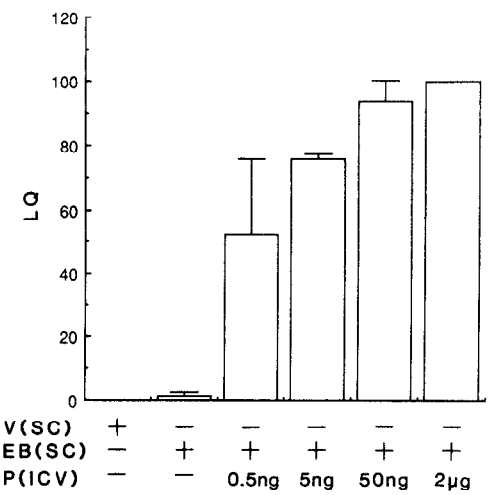

FIG. 1. Effects of varying doses of P on lordosis of female rats. Ovariectomized rats with indwelling stainless steel cannulas stereotaxically implanted into the third cerebral ventricle were injected sc with 10 μg EB in 0.1 ml sesame oil. Various doses of P (0.5–2 μg) in sesame oil were administered icv 48 h later. The animals were examined for their proceptive and receptive behaviors in the presence of a male 30 min and 3 h after P administration. The results of the experiments were expressed as the LQ, defined as the percentage of the number of complete lordosis responses by the female divided by the number of mounts by the male. The control rats received 0.1 ml vehicle (sesame oil; V), and another group received EB only. The *bars* represent the mean LQ ± SEM.

E-primed animals elicited reliable proceptivity and quantitative receptivity (measured and expressed as LQ).

Inhibition of behavioral responses by RU 38486 and ZK 98299

Intracerebroventricular injection of the antiprogestins RU 38486 and ZK 98299 suppressed P-facilitated behavioral responses in E-primed female rats at both 30 and 180 min. Both antagonists were very effective in suppressing lordosis when administered 1 h before P (Fig. 2). Hopping, darting, and ear wiggling were also completely inhibited by the antagonists. Again, all E-primed female rats treated with P alone displayed receptive responses compared to E-primed or vehicle-administered control rats.

Antisense and sense oligonucleotides and behavior

Sexual behavior was almost completely blocked in animals treated icv with the phosphorothioated antisense oligonucleotide to rat P receptor mRNA (PRAs) at a 4-nmol dose (Fig. 3). The females adopted aggressive posture toward the males and actively kicked off the males, and lordosis was very rare. Proceptive responses (ear wiggling and hop-darting) were reduced or completely suppressed in a dose-dependent manner (Fig. 3). In contrast, rats that received the sense phosphorothioated oligonucleotide (PRS) showed high levels of proceptive and receptive behaviors. Similarly, the nonspecific oligonucleotide (NS-O; Fig. 3) had no significant effect on P-facilitated sexual behavior. Both nonphosphorothioated sense and antisense oligos had little effect on lordosis regardless of the concentration administered (data not shown). Lordosis response and receptive behavior were maximally reduced only in animals that received oligonucleotides 0 and

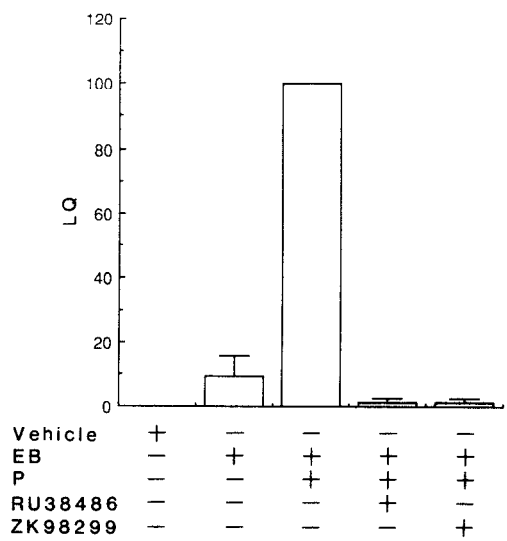

FIG. 2. Inhibition of P-facilitated sexual behavior in E-primed rats by P antagonists. Ovariectomized EB-primed (10 μg, sc) rats with stainless steel cannulas in the third cerebral ventricle were given icv injections of RU 38486 or ZK 98299 after 48 h. P (2 μg) was microinjected icv into the third cerebral ventricle 1 h later. The animals were tested for their proceptive and receptive behaviors 30 min and also 3 h after P administration, and the receptive behavior was expressed as the LQ, as described in Fig. 1. Values presented are mean ± SEM LQ.

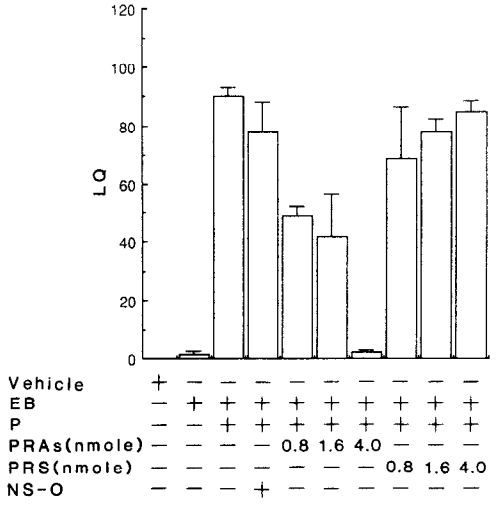

FIG. 3. Dose response of icv administered antisense (PRAs) and sense (PRS) oligonucleotides to PR mRNA on P-facilitated lordosis behavior in EB-primed rats (10 μg). Ovariectomized rats with indwelling cannulas in the third cerebral ventricle were injected with 0.8, 1.6, and 4 nmol of the oligonucleotides at the time of E priming. Animals were injected with the same doses of the oligonucleotides 24 h later and tested for lordosis response 30 min and 3 h after P administration. To another group of ovariectomized E-primed cannulated animals, nonspecific oligonucleotides (NS-O), consisting of a specific sequence for directional cloning into SP-6 vector, were administered in lieu of the other oligonucleotides. The animals were scored for sexual behavior, and the results are expressed as described in Fig. 1. Control animals received vehicle alone (V), E alone (EB), or P 48 h after E priming (E + P). The values presented are the mean ± SEM LQ.

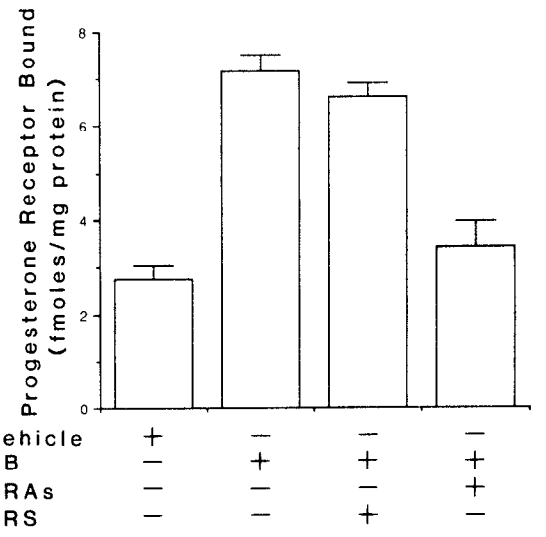

FIG. 4. Mean (±SEM) PR concentration in cytosol from the hypothalamus of ovariectomized rats with indwelling cannula injected sc with 10 μg EB. The antisense (PRAs) or sense (PRS) oligonucleotides to PR (4 nmol) were administered icv concurrent with EB and 24 h later. The animals were killed 48 h after EB administration, the hypothalamus was dissected and homogenized, and progestin binding assays were performed on the cytosol, as described in *Materials and Methods*. Each *point* is the mean of four to six independent determinations.

24 h after E priming, whereas a single administration at 0 h was only partially effective (data not shown).

Antisense and sense oligonucleotides and P receptor concentration in the hypothalamus

Estradiol priming induced a 161% increase in the concentration of hypothalamic cytosol PRs, which was significantly higher ($P < 0.001$) than that caused by the vehicle control treatments (Fig. 4). Infusion of antisense oligonucleotides to the PRs (PRAs; 4 nmol) caused a significant decrease (by 52.2%) in the estradiol-induced concentration of hypothalamic cytosol PRs ($P < 0.001$). Pairwise multiple comparision by the Student-Newman-Keuls method indicated a significant effect of antisense oligonucleotides on estradiol pretreatment ($P < 0.05$). However, infusion of sense oligonucleotides (PRS; 4 nmol) had no significant effect on the concentration of progestin receptors ($P > 0.05$).

Discussion

We have demonstrated that antisense oligonucleotides to PR mRNA administered via the third cerebral ventricle suppress P-facilitated sexual behavior in the female rat. As this treatment also reduced E-induced synthesis of PR in the hypothalamus, our results support the idea that PR is a critical component in the control of sexual behavior in the female rat.

The use of antisense RNA and DNA oligonucleotides to suppress gene activity has been successful both *in vivo* and *in vitro* (33, 34). These studies have shown that antisense oligonucleotides targeted to cellular RNA sequences can produce biological effects that have been correlated with reduced levels of RNAs or proteins. Similar effects on hormonally induced behavior have been observed by others using direct injections of antisense oligonucleotides to PR form B into the VMN (35, 36). Although in these studies, a reduction of the receptor protein was demonstrated in T47D cells *in vitro* (35) and by immunohistochemistry *in vivo* (36), in the present study we report a reduction in cytosolic PR in the hypothalamus, as quantified by *in vitro* binding assays. Although it is clear that treatment of our rats with antisense oligonucleotides to PR mRNA reduced the synthesis of PR in the hypothalamus, the mechanism of action of antisense oligonucleotides is not completely clear (37). Whether the antisense inhibition in the current study is due to transcriptional attenuation of mRNA or disruption of posttranscriptional processes, such as arrested translation and/or degradation of PR mRNA, is not known at present. It is surprising that a 52.2% decrease in the E-induced cytosolic PR seen in the presence of antisense oligonucleotides completely abolished the lordosis response. However, it has been shown that similar reductions induce a hyposensitive state (30). After the termination of the period of sexual receptivity in guinea pigs, a refractory period or a period of sequential inhibition occurs, during which the animals are hyposensitive and unresponsive to P. This P-induced hyposensitivity has been correlated to reduced cytoplasmic PR and a significant decrease in the accumulation of nuclear PR in the hypothalamus (30, 38). This P-induced hyposensitivity could be overcome by administering estradiol or a large dose of P, resulting in nuclear accumulation of PR (30). Thus, it is apparent that

a threshold concentration of PR is an absolute requirement for P-facilitated lordosis, and a decrease in the concentration, but not a complete loss of the receptors, by the antisense oligonucleotides may have offset the facilitatory influence of P. The importance of threshold levels of inducible PR in the mediobasal hypothalamus-preoptic area for the expression of feminine sexual behavior has also been reported (39, 40).

Several investigators have suggested that E-induced PR is a prerequisite for P-facilitated sexual behavior (39–41). The P antagonist RU 38486 suppresses P-facilitated reproductive behavior in a dose-dependent manner when administered 1 h before P (42–45), and it abbreviates the period of sexual behavior when administered after P (45, 46). Furthermore, the antagonist appears to be a competitive inhibitor of hypothalamic-preoptic area progestin receptor binding, as demonstrated by hormone exchange assays (43, 45). Inhibition of the sexual behavior of E-primed animals by icv administration of RU 38486 1 h before P in the present study is in accordance with these reports. As RU 38486 also blocks glucocorticoid and androgen actions, it can be argued that such results are not conclusive. However, in our study we used the P antagonist ZK 98299, which is thought to have no antiglucocorticoid activity (47), to block P-induced sexual activity. These data with ZK 98299 and our results with the antisense block of PR synthesis strongly support the idea that PR plays a critical role in P-facilitated sexual behavior in the female rat.

The display of proceptive behavior in E-primed animals within 30 min after icv administration of P could be due to a direct action of P on the neuronal membranes. It has been proposed that rapid behavioral effects facilitated by P may be nongenomic and membrane receptor mediated (48). This is supported by the observations that lordosis behavior could be induced within 10 min after iv, intracerebral, or intraventricular administration of P to E-primed animals (49–52). The specific binding of P and other steroids to synaptic plasma membranes adds to the possibility that P acts to facilitate lordosis by interaction with cell surface receptors (53–56). On the other hand, these effects may involve early genomic responses that occur within minutes after hormone exposure. Such hormone-induced response patterns have been observed in the uterus (57–59). Thus, the mediation of P's action on lordosis could involve a combination of nongenomic membrane mechanisms as well as genomic actions via intracellular receptors and/or their interaction.

In summary, E acts at the level of nuclear DNA to increase the expression of the PR gene in the VMN of hypothalamus. P-facilitated lordosis behavior is mediated by P activation of its receptor. Interruption of PR gene expression using antisense oligonucleotides inhibits P-facilitated female sexual behavior. Thus, the present data support the notion that steroid hormones initiate a cascade of events at the genomic level in the VMN of the hypothalamus to regulate the neuronal networks involved in the control of female sexual behavior in rats.

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