Dopamine Requires the Unoccupied Progesterone Receptor to Induce Sexual Behavior in Mice

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Using the recently generated mutant mice strain (PRKO) carrying a null mutation for the progesterone receptor (PR) gene by gene targeting, we examined the critical role of PR as a coordinator of key regulatory events involved in the steroid hormone and dopamine-facilitated sexual behavior in female mice. *In vitro* one-point binding analyses of estradiol benzoate (EB)-induced cellular PRs and immunohistochemistry of PR in the mediobasal hypothalamus demonstrated a reduction in binding in the homozygous females, equivalent to background levels seen in EB-unresponsive tissue. The biochemical findings correlated well with the behavioral observations, with the wild type females exhibiting high levels of lordosis, while the homozygous females showed minimal lordosis in response to mating by male mice. As a critical validation of our earlier studies on ligand-independent activation of PRs by dopamine, we examined the facilitation of sexual behavior by a dopamine agonist in the null mutants. Wild type females having the full complement of PRs exhibited high levels of lordosis, while the homozygous females showed minimal lordosis in response to dopamine. To determine whether this reduced response was due to a general lack of ability to express lordosis, mice were treated with another neurotransmitter, serotonin. No significant difference in the serotonin-facilitated lordosis response was observed between the wild type and the homozygous females. We conclude that multiple signal transduction pathways coexist in the neuroendocrine system for reproductive behavior, with PR acting as a transcriptional mediator for dopamine, as well as progesterone, to achieve integration of neural communication in the central nervous system. (Molecular Endocrinology 10: 1728–1737, 1996)

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INTRODUCTION

We have reported previously that certain steroid receptors can be activated to induce gene expression in cultured cells in the absence of their cognate ligands (1). The neurotransmitter dopamine (DA) and select peptide growth factors appear capable of such ligand-independent activation of sex steroid receptors (2, 3). In studies using antisense oligonucleotides to progesterone receptor (PR) mRNA delivered into the third intracerebral ventricle of rats, we reported elimination of *in vivo* reproductive behavior, lordosis, in response to both progesterone (P) and DA (4). To definitively show that the unoccupied PR is required for DA-induced sexual behavior *in vivo*, we turned to a genetically altered mouse model (5).

In ovariectomized female mice, as in other rodents, exogenously administered estradiol benzoate (EB) followed by P is effective in inducing sexual receptivity (6–8). Although a critical role for hypothalamic PR in the induction of sexual receptivity has been established using PR antagonists (9–11) and antisense oligonucleotides (11–13) in rats and guinea pigs, less is known about its role in the mouse, a species in which sexual receptivity is influenced by genotypic and strain differences in addition to experiential factors (8, 14). We have recently reported the generation of a PRKO (PR

knockout) mouse carrying a null mutation for the PR gene using gene targeting strategy (5). Preliminary studies demonstrated that adult female mice homozygous for the PR mutation did not exhibit high levels of lordosis in the presence of sexually experienced wild type males (5). We now report the results of studies examining the role of PR as a modulator of sexual receptivity in these transgenic mice. We present evidence for a crucial role for PR as a ligand-dependent transcription factor in the dopaminergic induction of sexual behavior in female mice. The importance of EB-induced neural PR for the EB + P induction of receptivity in mice is demonstrated in the correlation between receptor concentration and lordosis response. We also substantiate the ligand-independent activation of PR by DA (4) and provide further information on the role of PR as a mediator of the behavioral effect of this neurotransmitter in the facilitation of female sexual receptivity in mice.

RESULTS

Hormonal Induction of Sexual Receptivity in Parental Strains C57BL/6 and 129SvEv Female Mice: Effects of Dose Response and Hormonal Priming and Testing Experience

Since strain and genotypic differences with respect to hormonal induction of sexual receptivity are known to exist in female mice (7, 8), we performed a baseline study on the hormone inducibility of sexual behavior in the parental strains (C57BL/6 and 129SvEv) used to generate the PR mutant mice (C57BL/6 × 129SvEv). C57BL/6 and 129SvEv female mice were tested for sexual behavior in the presence of C57BL/6 and 129SvEv male mice, respectively. As shown in Fig. 1, A and C, EB alone was not capable of inducing high levels of lordosis in either strain at any of the doses tested. The administration of P, 48 h post-EB priming, resulted in high levels of sexual receptivity in both

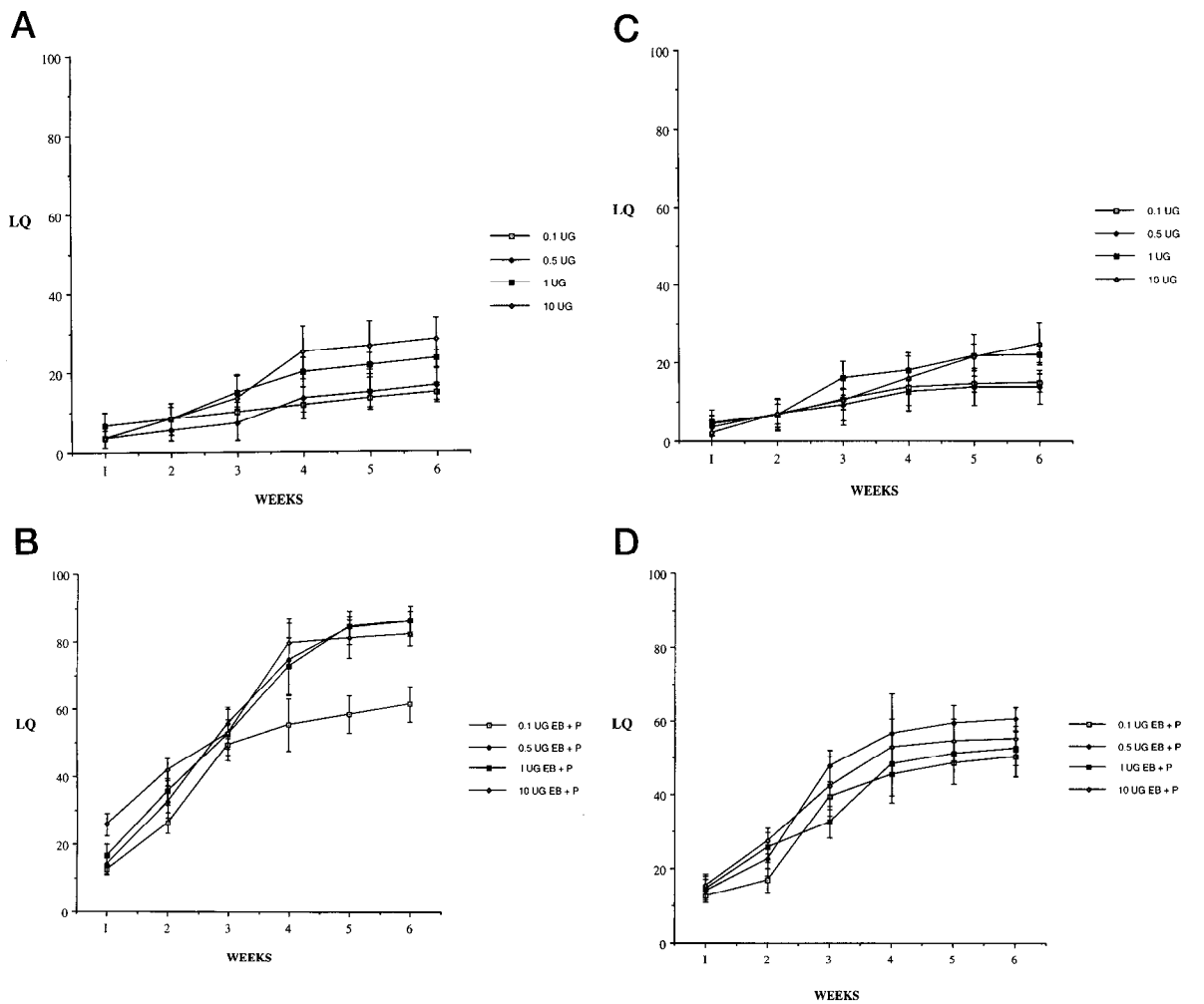


Fig. 1. Hormonal Induction of Sexual Receptivity in Ovariectomized C57BL/6 (A and B) and 129SvEv (C and D) Female Mice

All females were administered indicated doses of EB alone (A and B) or EB followed by P (100 μg) 48 h later. The hormones were administered each week for 6 weeks and tested weekly for sexual receptivity as described in *Materials and Methods*. Values are represented as mean LQ (lordosis responses/mounts × 100) ± SEM (n = 6–10 animals for each group).

strains. Although the facilitatory effects of P were similar at the higher doses of EB employed (0.5–10 μg), the lordosis quotients (LQs) for the groups primed with the lower dose of EB (<1 μg) were low during the entire period tested (Fig. 1, B and D).

Notably, both strains showed improved receptivity after weekly priming with EB and testing as reflected in the increase in LQs over time (Fig. 2). Strain differences were also evident, with the C57BL/6 females responding better at higher levels of EB + P stimulation than the 129SvEv females (Fig. 2). The data included in Figs. 1 and 2 should be a very useful to investigators who wish to employ the mouse model for studies of sexual behavior.

PR Levels in the Hypothalamus in the PR Mutants: Correlation with Sexual Receptivity

Because of tissue availability, only one-point binding analyses of cellular PRs in the medial basal hypothalamus (MBH) were carried out. A significant reduction in binding in the homozygous females (PRKO) compared with the wild type was observed (Fig. 3A). The binding to ^{3}H-labeled R5020 in the MBH was equivalent to that seen in EB-unresponsive tissue, *i.e.* the cerebral cortex, suggesting that the residual binding could be due to low affinity-binding protein. Heterozygote females had 40% less binding than the wild types. One-way ANOVA detected a statistically significant overall effect ($P < 0.0001$) in receptor levels. Pairwise multiple comparison by the Student-Newman-Keuls method indicated significant changes in the receptor levels of both the homozygotes compared with the wild types ($P < 0.001$) and in the heterozygotes compared with the wild types ($P < 0.01$).

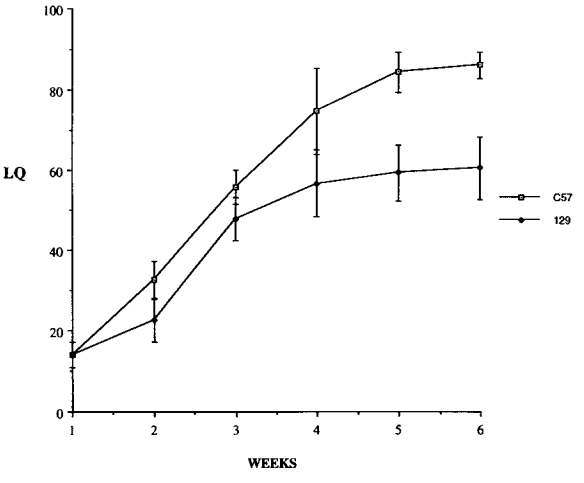


Fig. 2. EB-P Induction of Sexual Receptivity in Ovariectomized C57BL/6 and 129SvEv Female Mice

Ovariectomized female mice were sc administered EB (0.5 μg), followed by P 48 h later. The hormones were administered every week for 6 weeks and tested weekly for sexual receptivity 6 h after P administration. Values presented are mean LQ ± SEM (n = 6–8 animals for each group).

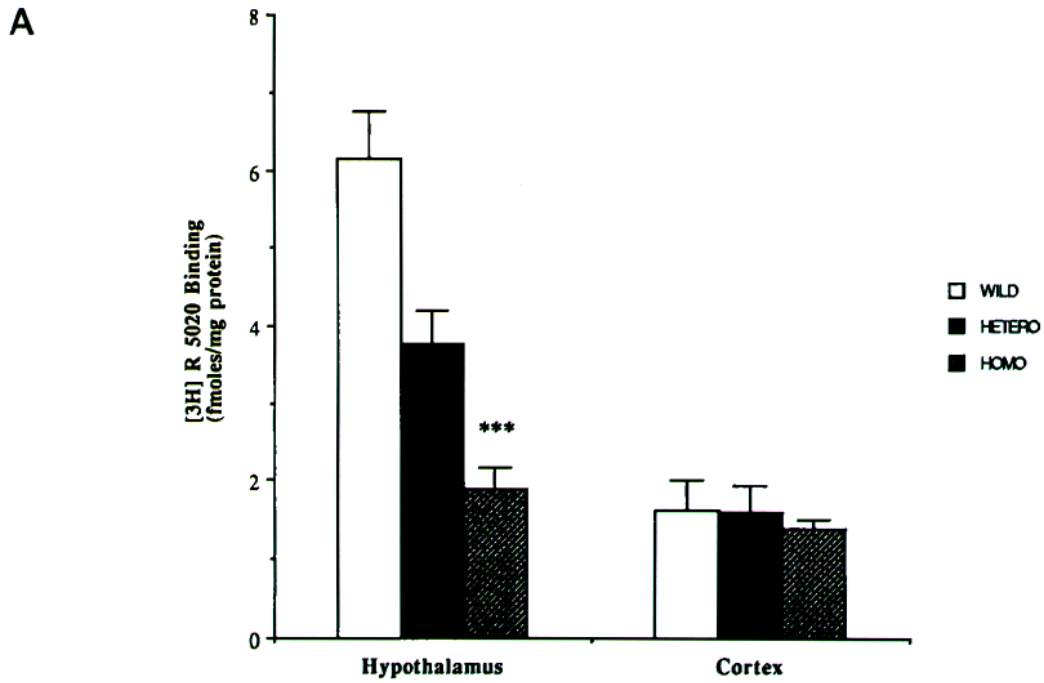
The [^{3}H]R5020 binding levels in the cerebral cortex did not attain statistical significance between the groups and likely represent assay background or low affinity binding. As an important substantiation of our interpretation, immunocytochemical analysis of free floating tissue sections in the region of hypothalamus indicated no detectable immunoreactivity in the homozygous female compared with the wild type female (Fig. 3B). P binding analyses in other tissues (*e.g.* uterus, breast, etc.) also revealed no PR mRNA or protein in the PRKO animals.

Ovariectomized PRKO female mice injected with EB + P and tested for sexual behavior in response to mating by wild type male mice exhibited distinct genotypic variations in lordosis response, roughly correlating with the hypothalamic PR levels (Fig. 4A). Both wild type and heterozygous females exhibited high levels of lordosis, while homozygous females showed a nonsignificant response. EB alone was not sufficient to induce receptivity in any of the mutants tested (Fig. 4A). Kruskal-Wallis one way ANOVA on ranks indicated a statistically significant overall effect ($P < 0.0001$) of hormone treatment in all groups. Dunn's method of multiple comparisons indicated statistically significant ($P < 0.05$) differences in the lordosis response between EB + P-treated homozygote and heterozygote animals compared with wild type animals treated with EB + P. Repeated EB + P priming and mating experience resulted in inducing high levels of lordosis in wild type and heterozygous mice over a period of 3–5 weeks (Fig. 4B), a response pattern typical for mice (7, 14). Similar treatment had no effect on the receptivity of the PRKO animals, as shown by the display of minimal responsiveness by these females throughout the period tested (Fig. 4B).

Unoccupied PRs are Involved in Mediating DA Effects on Female Sexual Behavior

As a prelude toward examining the central *vs.* peripheral actions of compounds, we compared the facilitatory effects of P, administered intracerebroventricularly (icv) *vs.* subcutaneosly (sc), on lordosis response in EB-treated females in the presence of wild type male mice. As seen in Fig. 5, icv administration of P into the third cerebral ventricle, via a chronic indwelling cannula, produced high levels of receptive behavior comparable to sc injection of P, in wild type mice on week 6 of testing. LQs were high in P-treated animals compared with EB-treated animals at all doses (0.5, 1, 100 μg; $P < 0.01$).

In earlier studies, we demonstrated the requirement for PR in mediating the effects of DA on female sexual behavior in EB-primed rats by transiently suppressing PR levels using antisense oligonucleotides to PR. To definitively substantiate this hypothesis, the facilitation of sexual behavior by a DA receptor (D1) agonist, SKF 38393, was examined in the PR null mutants. The effects on lordosis response (LQ) of different doses of



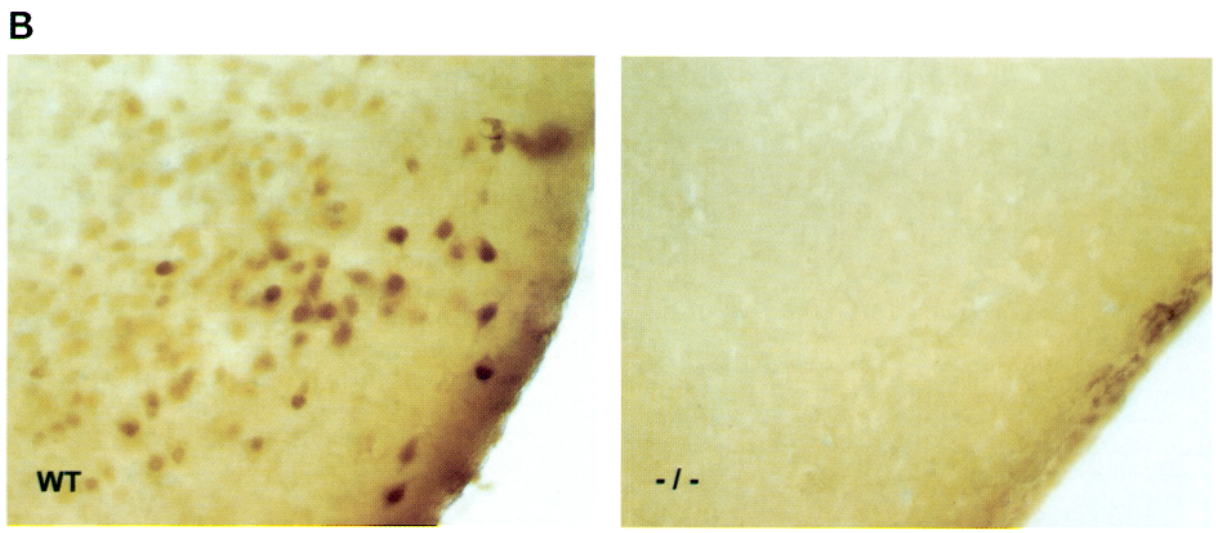


Fig. 3. PR Immunoreactivity in the Hypothalamos of Wild Type and PRKO Mice

A, PR levels in the hypothalamus of PR mutants. Mean (±SEM) PR concentration in cytosol from the hypothalamus and cortex of ovariectomized mice, injected sc with 0.5 μg EB. The animals were killed 48 h after EB administration, the hypothalamus and cortex were dissected and homogenized, and progestin binding assays were performed on the cytosol as described in *Materials and Methods*. Each point is the mean of six independent determinations. ***, $P < 0.001$. B, PR immunoreactivity in the ventromedial hypothalamus. Using diaminobenzidine-peroxidase technique, PR was detected in the hypothalamus of ovariectomized and estrogen-treated wild type (WT) mice. However, PR immunorectivity was not detected in hypothalamic areas of PR mutant (−/−) female mouse treated under identical conditions. Images were obtained through 20 × objective on a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) and captured in digital format through a Hamamatsu C5810 camera. Images are acquired under identical optical conditions and were transferred to Adobe Photoshop 3.0.

D_1 agonist, administered icv, was examined in EB-primed wild type PR mice (Fig. 6A). At a dose range of 10–50 ng of the D_1 agonist, more drug-injected females exhibited lordosis responses ($P < 0.001$) compared with controls ($P < 0.001$) beginning within 30 min of injection and lasting for at least 2 h. At doses higher than 100 ng, the mice appeared less active compared with the controls, and toxicity was suspected ($P > 0.05$). Comparison of the facilitatory effects of SKF 38393 (50 ng) on lordosis response in EB-primed, wild type *vs.* homozygous null females demonstrated no enhanced lordosis ($P < 0.001$) in the PR-deficient homozygotes compared with EB alone (Fig. 6B).

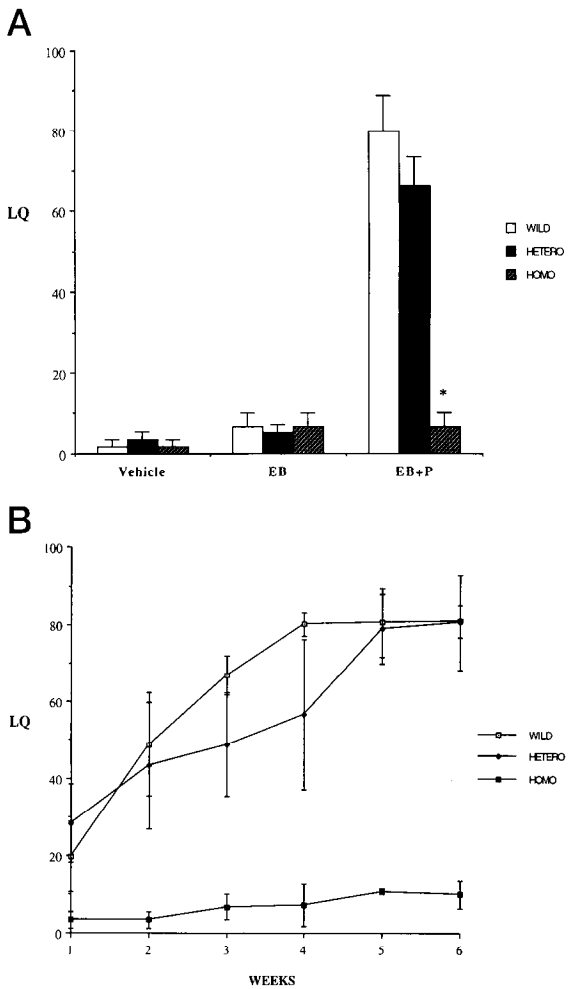


Fig. 4. EB + P Induction of Sexual Receptivity in PR Mutants

A, Ovariectomized mice were administered 0.5 μg EB, followed by P (100 μg) 48 h later. The hormones were administered every week for 6 weeks and tested weekly for sexual receptivity in the presence of wild type PR male mice. Values are presented as mean LQ ± SEM from week 4 of testing. Statistically significant (*, $P < 0.05$) differences were observed in EB + P-treated homozygotes as compared with wild type animals that received the same treatments. B, Effect of weekly hormonal priming and testing on lordosis response in PR mutants. Ovariectomized mice from the three genotypes were primed weekly with EB + P as described in panel A and tested weekly for sexual receptivity. By week 5, both heterozygotes and wild type mice showed equal responsiveness while homozygotes exhibited minimal responsiveness (n = 6 for all groups). Values are presented as mean LQ ± SEM. Control groups of mice received vehicle (veh) or EB treatments.

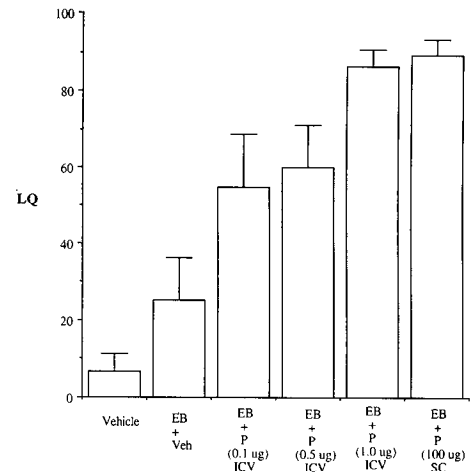


Fig. 5. Dose Response of P on EB-Primed Wild Type PR Mice

Ovariectomized female wild type mice were weekly primed with EB (0.5 μg) and P (100 μg) and tested weekly for sexual receptivity in the presence of wild type PR male mice for 4 weeks. On week 5, stainless steel cannulae were implanted stereotaxically into the third cerebral ventricle. On week 6, the cannulated animals were injected sc with 0.5 μg EB, followed by icv administration of P (0.1–1 μg) or sc injection of P (100 μg). The animals were tested for sexual receptivity in the presence of wild type PR male mice, 30 min after icv administration or 6 h after sc administration of P. Control mice received vehicle or EB treatments. Values are presented as mean LQ ± SEM. Statistically significant effects (**, $P < 0.001$) were observed in EB-primed mice that received 1 μg P, icv, and 100 μg P, sc (n = 6 in each group).

Serotonin Mediation of Lordosis Response Is Not Dependent Upon PR

Serotonin has been reported previously to stimulate lordosis in EB-primed rats (15). A dose-response curve of serotonin indicated that the EB-primed wild type female mice exhibited high levels of lordosis at a dose of 50 ng serotonin (icv) *vs.* EB-treated controls ($P < 0.05$) (Fig. 7A). There was no significant difference ($P > 0.05$) in the facilitatory effects of serotonin on the lordosis responses of the EB-primed wild type and the homozygotes, indicating that the effects mediated by serotonin are not dependent upon the presence of PR (Fig. 7B). This experiment is informational in two respects. First, it indicates that at least one other parallel pathway exists for sexual behavior that does not converge via PR. Second, it demonstrates that the PRKO animals do not have a developmental defect that prevents induction of sexual behavior *per se*.

DISCUSSION

Using a transgenic mouse carrying a null mutation of the PR gene, we have shown an important physiological role for PR as a coordinator of key regulatory events involved in steroid hormone and neurotransmitter-facilitated sexual behavior in female mice. By demonstrating a correlation between receptor concentration in the hypothalamus and the behavioral responsiveness in the different genotypes, we have substantiated the requirement for elevated levels of PR for responsiveness to P in mice. The inability of both P

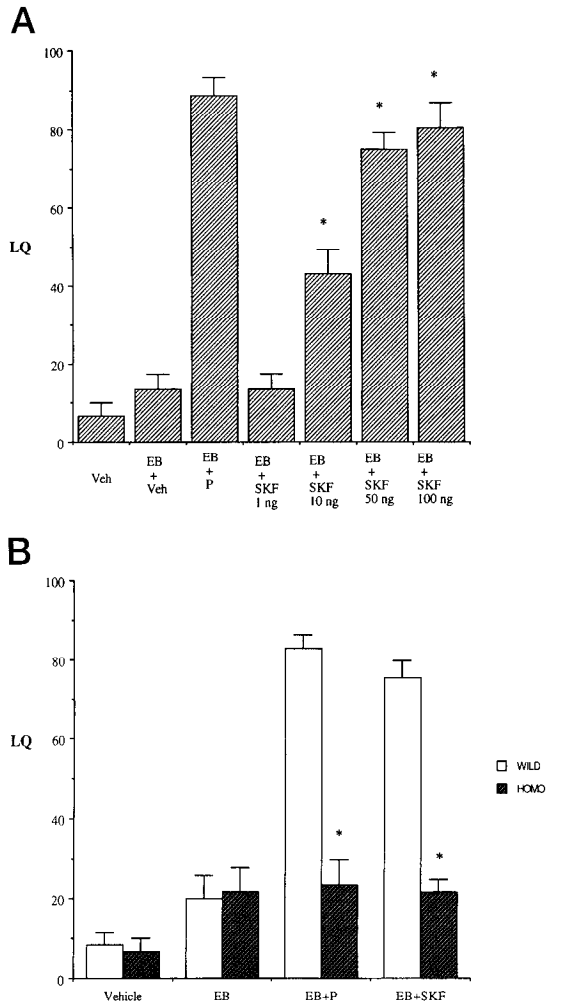


Fig. 6. Dose Response of icv Administered DA D_1 Agonist, SKF 38393 (SKF) on Lordosis Response of PR Mutants in the Presence of Wild Type PR Male Mice

A, Ovariectomized wild type PR transgenic mice were primed weekly with EB + P and tested weekly for 4 consecutive weeks as described in *Materials and Methods*. On week 5, stainless steel cannulae were implanted chronically into the third cerebral ventricle. Varying doses of SKF 38393 (1–200 ng) were administered icv 48 h after EB priming; sexual behavior of the mice was observed 30 min later. Control groups of animals received vehicle (vehisaline) or EB followed by saline or P 48 h later. Statistically significant differences were observed (**, $P < 0.01$) in SKF-treated mice compared with the EB-treated controls. B, Ovariectomized homozygotes and wild type PR mutants were primed and tested weekly as described in panel A. After implantation of cannulae on week 5, the mice were primed with EB on week 6 and tested for sexual behavior 30 min after icv administration of D_1 agonist, SKF 38393 (50 ng). Control mice received vehicle (saline) or EB followed by saline or P 48 h later. Statistically significant differences were seen in SKF and P-facilitated responses of the homozygotes compared with the wild type (*, $P < 0.001$) (n = 6 for each group).

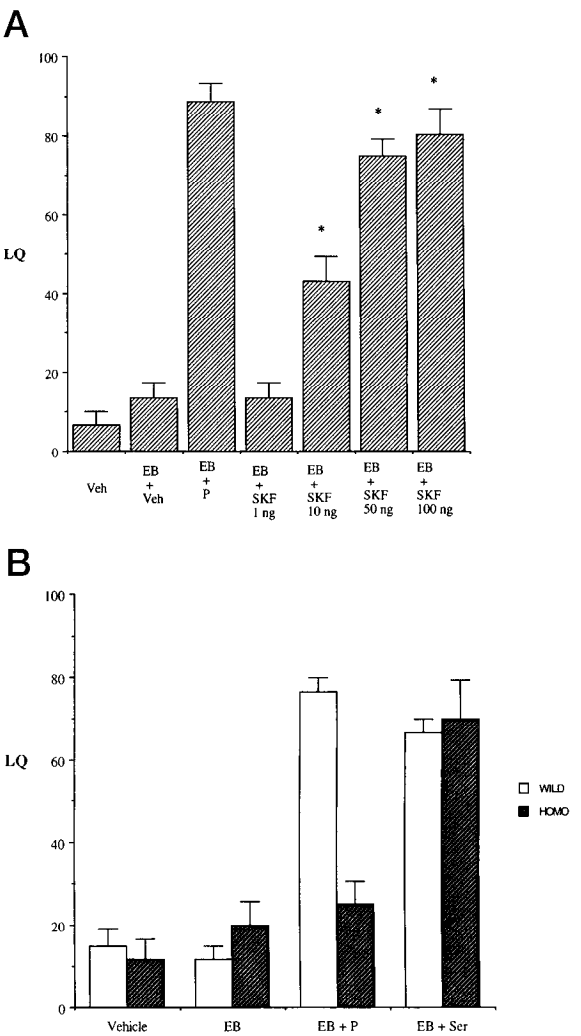


Fig. 7. Dose Response of icv Administered Serotonin (Ser) on Lordosis Response in PR Mutants in the Presence of Wild Type PR Male Mice

A, Ovariectomized wild type PR mice were subjected to weekly hormonal priming and behavioral testing as described for Fig. 5A. Third cerebral ventricle cannulations were performed on week 5, followed by EB priming on week 6 and icv administration of serotonin (1–200 ng) 48 h later. The mice were tested for sexual responsiveness 30 min after icv administration of serotonin. Controls were the same as described for Fig. 6. A significant effect of serotonin was seen at a dose of 50 ng as compared with the EB-treated controls (*, $P < 0.05$). B, The effects of serotonin on lordosis response of homozygotes and wild type PR mutants was examined using the paradigm described in Fig. 6B. The EB-primed mice received serotonin (50 ng), 48 h after EB administration. Controls were the same as described for Fig. 6. No significant differences were seen in serotonin-facilitated responses between the wild type and homozygous PR mutants. ($P > 0.05$) (n = 6 animals in each group).

and the neurotransmitter DA to facilitate sexual behavior in PR null mutants now provides definitive evidence to substantiate our earlier studies demonstrating cross-talk between DA and PR and extends the findings to another rodent species.

Similar to other rodents, hormonal induction of sexual receptivity in ovariectomized female mice can be

reinstated by the sequential treatment with EB and P (16). While estradiol alone restores a minimal level of sexual receptivity, it interacts synergistically with P to restore high levels of receptivity. The present data clearly indicate that regardless of EB dose, minimal receptivity is evident in animals when P is not coadministered, consistent with other reports on induction of sexual receptivity in the mouse (6). Although, the female members of a variety of rodent species appear similar in terms of the hormonal manipulations necessary for induction of sexual behavior, mice appear to differ from other rodents in one aspect. While rats can achieve maximum levels of receptivity after one or two EB + P treatments, mice require sequential treatments and testing experience to achieve a good receptivity response (6, 7) regardless of the strain. A reliable protocol for studying mouse reproductive receptivity has been generated in the course of these studies.

Studies on the two parental strains, C57BL/6 and 129SvEv, from which the mutant mice were generated, indicated moderate differences in their behavioral responsiveness to exogenous hormonal stimulation similar to that observed in other strains (14). While the C57BL/6 mice were highly responsive by 3–5 weeks of priming and testing, the 129SvEv strain exhibited a slightly slower rate of development and a maximal response of a 60% LQ. Nevertheless, both strains required at least 4 weeks of repeated hormone treatment and testing to achieve their maximal level of responsiveness. A similar trend was observed in all the experimental groups of mice, enabling us to perform our studies at this time point.

Several studies have demonstrated that P-facilitated lordosis behavior in rodents is mediated by the activation of its intracellular receptors in the MBH and preoptic area (17–19). The concordance between receptor levels and behavioral responsiveness observed in the present study is in agreement with these findings. Both wild type and heterozygous females exhibited high levels of receptivity while the homozygous knockouts failed to achieve minimal lordosis response. The behavioral and biochemical findings of these experiments reveal the importance of EB-induced PR in the MBH-preoptic area for the expression of female sexual behavior, confirming earlier reports (11, 13, 16, 17, 20).

A nongenomic mechanism underlying P facilitation of sexual behavior has been proposed previously. It was based upon the very rapid observable effects after hormone exposure (21–25). The minimal responsiveness exhibited by the homozygotes argues against membrane-bound PR being the primary mediator of lordosis response in mice. However, we cannot rule out the possibility that nongenomic membrane mechanisms play a collaborative role with genomic mechanisms. Possible relationships between membrane-bound and intracellular PR in mouse sexual behavior require further clarification.

In earlier studies using antisense oligonucleotides to PR, we demonstrated a novel mechanism by which intracellular PR could mediate the effects of DA on female sexual behavior in rats (11). Consistent with these findings, icv administration of a D_1 agonist facilitated high levels of lordosis in wild type female mice containing a full complement of PR, while the homozygous females lacking EB-induced PR showed low levels. While the present data clearly demonstrate that PR is required for at least one behavioral aspect of DA in female mice, its role in other active and passive behavioral components of sexual behavior (26) needs further examination. In this context, it is worthy to note that a significant increase of DA is seen in the nucleus accumbens and striatum (27) concurrent with DA release in the ventromedial hypothalamus region of female rats during copulation (28), suggestive of its association with aspects of sexual behavior other than lordosis.

The mechanism of action of another neurotransmitter, serotonin, in the facilitation of lordosis response was also examined in our PR null mice. Previous studies in rats have suggested both inhibitory and facilitatory effects of serotonin on lordosis response (15, 29–38). Our own results clearly suggest a facilitatory role of serotonin on lordosis in mice in agreement with similar findings in rats (32, 36, 37). It is possible that the lordosis-facilitating effects of serotonin in mice are mediated by a subpopulation of serotonin receptors as has been observed in rats (39, 40). The wild type and homozygote female mice exhibited similar facilitatory responses, indicating that the serotonin-mediated response does not require intracellular PR. This result is similar to our own unpublished observations in rats, in which the response to serotonin was not suppressed by antiprogestins or by antisense oligonucleotides to PR mRNA given icv. Importantly, this result also indicates that PR null mice are still capable of exhibiting lordosis and do not have a developmental defect that prevents a sexual behavior response. The data also suggest that the membrane receptor-dependent pathway initiated by serotonin does not converge with the intracellular PR pathway by which P and DA appear to facilitate sexual behavior, proving redundancy in sexual response pathways in the central nervous system.

It is possible that different neurotransmitters modify behavioral responsiveness to steroid hormones in rodents by affecting steroid-dependent gene transcription events differentially. Since protein phosphorylation is common to the pathways and molecular mechanisms through which both neurotransmitters and steroid hormones produce their biological effects (41, 42), it is plausible that multiple signal transduction pathways interact (or synergize) to achieve the eventual behavioral response. While steroid hormone receptors function as general transcription factors for neural communication by the dopaminergic system, perhaps by altering the second messenger systems or neuronal phosphoproteins (41, 43), other functional second messenger systems may reinforce the steroid-

initiated response to achieve the integration required for highly complex neuroendocrine behavior.

MATERIALS AND METHODS

Female mice of C57BL/6 and 129SvEv strains were commercially obtained from Taconic Farms (Germantown, NY) at about 4 weeks of age. Age-matched wild type heterozygote and homozygote PR mutant female mice were generated and obtained from the breeding colony at Baylor College of Medicine. Eight-week-old male mice, belonging to strains C57BL/6 and 129SvEv, were obtained from Taconic Farms, and PR wild type male mice were bred in the laboratory. All animals were maintained on a 12-h light, 12-h dark reversed light cycle with lights off at 1200 h and food and water *ad libitum*, and their care was in compliance with Federal guidelines.

Procedures

All female mice used in this study were ovariectomized under avertin (10 mg/ml) anesthesia at least 2 weeks before any hormonal manipulation. Weekly hormone administrations began 2 weeks after surgery. All hormones were dissolved in sesame oil and were injected sc unless specified. EB was administered 48 h before 100 μg P, and behavioral testing was done in the presence of males 6 h later. Dose response studies with EB±P were performed on different groups of females from the strains mentioned. For 6 consecutive weeks, females received weekly injections of hormones and were tested weekly for sexual receptivity. All steroids were purchased from Sigma Chemical Co. (St. Louis, MO).

Behavioral Studies

All behavior tests were conducted during the dark phase of the reversed light-dark cycle. A single test of receptivity involved placing the female mouse into the home cage of a male mouse of proven sexual vigor and of the same strain as the female. The female mice derived from parental mouse strains C57BL/6 and 129SvEv were tested in the presence of C57BL/6 and 129SvEv male mice, respectively. The wild type, heterozygote, and homozygote PR mutant female mice derived from the two parental mouse strains (C57BL/6 and 129SvEv) were tested in the presence of wild type PR male mice derived from the same genetic background (C57BL/6 × 129SvEv). The test was continued until the male had mounted the experimental female 10 times. Only mounts in which the male showed pelvic thrusting were scored. If the male did not mount the female during the testing period, she was placed with a different male. The female's response to a mount was categorized as a lordosis response when it displayed a rigid posture with arching of the back, elevation of the hind quarters, and deviation of the tail to facilitate male mounting and intromission (7, 44, 45). Failure to achieve lordosis was typified by a lack of positional response or by other responses, such as vocalization and active attempts by the female to escape, or by complete immobility with the hind quarters down. LQ was calculated as a percentage of the total number of lordosis responses divided by the total number of mounts. It was used as a measure of sexual receptivity. The experimental observer was blind to the mouse genotype and hormonal treatment conditions until behavioral measurements were completed.

Stereotaxic Surgery and Central Administration of Compounds

A stainless steel guide cannula (26 gauge) was implanted adjacent to the VMN of the hypothalamus into the third ventricle of the anesthetized female mice using a Lab Standard stereotaxic instrument (Stoelting, Wood Dale, IL). The coordinates used for the third ventricle were: antero-posterior, bregma −1.5 mm; lateral, just on the midline (above the longitudinal sinus); and dorsoventral, −5 mm according to stereotaxic atlas (46). A dummy cannula (33 gauge) filled and sealed the guide cannula when not in use. The surgical tools were obtained from Plastics One (Roanoke, VA) Stereotaxic implantation of the cannula was done on week 5 of repeated hormone priming and behavioral testing. Varying doses of P in sesame oil, DA agonist SKF 38393, or serotonin in saline were administered icv at 48 h after EB priming, beginning 1 week after cannulation. Control animals received similar injections of vehicle only. The animals were tested for receptive behavior beginning at 30 min after the icv administration.

Cytosol PR Assays

EB-induced hypothalamic cytosol PR were assayed by one-point binding analyses as described previously (11, 47). All the steps were carried out at 0–4 C. The MBH was dissected out, caudally by the caudal edge of the mamillary bodies and rostrally by the caudal edge of the optic chiasm. Diagonal cuts were made extending from the hypothalamic fissures to the midpoint of corpus callosum forming lateral boundaries and a cut below the level of 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 high speed supernatant were incubated with 0.4 nM [^{3}H]R5020 final concentration (specific activity 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]R5020 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, Downer's Grove, IL). The concentrations of cytosol protein were assayed by the method of Bradford (48), and the data presented as femtomoles of [^{3}H]R5020 specifically bound per mg of protein. Cytosol PR from the cortex was measured for reference comparison. Actual numbers were presented in all cases.

Immunocytochemistry

Ovariectomized, EB-primed animals were deeply anesthetized and perfused transcardially with heparinized PBS followed by fixative (2% picric, 4% paraformaldehyde in PBS). The brains were removed and incubated in fixative overnight. The fixative was then replaced with gradient of buffered sucrose (10%, 20%, and 30%, 24 h each), and coronal sections (50 μm) were prepared using sliding microtome.

Immunocytochemistry was performed on free-floating sections as described previously (49). Briefly, brain slices were washed in PBS and incubated with hydrogen peroxide followed by 10% normal goat serum to diminish nonspecific binding for 1 h. Monoclonal anti-PR antibody (4 mg/1 ml, Affinity Bioreagents, Inc., Neshanic Station, NJ) were diluted in PBS containing 0.03% Triton X-100 and incubated for 48 h at 4 C. Sections were incubated with biotinylated second antibody (1 h at room temperature), and antigen was visualized using Histostain SP Kit (Zymed Laboratories, Inc., San Ramon, CA). PBS was used to wash sections for 30 min between all steps (minimum three changes).

Data Analysis

Statistical analysis was done by either of the methods as appropriate: For each significant ANOVA, post-hoc comparisons were made using Dunnett's or the Student-Newman-Keuls method, or Kruskal-Wallis one-way ANOVA on ranks method of analysis was followed by Dunn's method for comparison. InStat (Graph Pad) was used.

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