

Hypothalamic progesterone receptor-A mediates gonadotropin surges, self priming and receptivity in estrogen-primed female mice

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Abstract

Ovarian progesterone (Prog) is an essential steroid hormone for the secretion of GnRH and reproductive behavior. It exerts primary effects through the progesterone receptor (PR). When analyzed separately *in vitro*, PR isoforms (PR-A, PR-B) display striking differences in transcriptional activity. The present study was undertaken to determine the *in vivo* impact of each isoform on hypothalamic function in female mice with ablation of a single isoform, either PR-A or PR-B. To this end, we used single-cell RNA analyses, reverse transcriptase real-time (q)PCR mRNA analyses of punched-out tissue, immunohistochemistry, and reproductive behavior. We provide evidence for the requirement of PR-A in individual ventrolateral ventromedial nucleus (vVMN) neurons for Prog-facilitated proceptive and receptive behaviors in estrogen benzoate (EB)-primed females and the reciprocal male interactions. We clarify histological and molecular mechanisms of PR isoform activity by showing that (1) PR-A is predominant in individual vVMN neurons controlling female lordosis circuitry, whilst (2) PR-B is predominant in those VMN subdivisions that provide for amplification of PR-A activity. We go on to demonstrate that PR-A is dominant in the anteroventral periventricular nucleus but not the arcuate nucleus that feed fibers into and around the VMN. In the medial preoptic area, high levels of GnRH RNA in EB-primed PR-A-expressing mice were seen coincident with increased plasma LH levels. Two consecutive GnRH pulses enhanced LH only in primed PR-A-expressing females. In all, the findings are consistent with the hypothesis that hypothalamic PR-A-mediated genomic activities result in reproductive behavior coordinated with ovulation.

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Introduction

The ovarian steroid hormone progesterone (Prog) is important for female reproductive functions, including ovulation, uterine and mammary gland development, maintenance of pregnancy, and sexual behavior (Clarke & Sutherland 1990, Lydon *et al.* 1995). The physiological effects of Prog are mediated by interaction with cognate intracellular receptors (PR) of which there are two isoforms (PR-A, PR-B; Conneely *et al.* 1987, Kastner *et al.* 1990, Kraus 1993). As a member of the large nuclear receptor superfamily of transcription factors (Evans 1988, Mangelsdorf *et al.* 1995), PR regulates gene transcription by binding to DNA-regulatory sequences and by specific interactions with coregulator proteins for activation of the RNA polymerase complex (McKenna *et al.* 1999). PR is encoded by a single gene subsequent to transcription from two alternative promoters and initiation of translation at two alternative AUG initiation codons (Conneely *et al.* 1987, Kastner *et al.* 1990, Kraus 1993).

The structural difference between PR-A and PR-B isoforms is an additional stretch of 174 amino acids located at the amino-terminal in the PR-B isoform. This

segment encodes a transactivation function (AF3) specific to the PR-B isoform (Sartorius *et al.* 1994, Wen *et al.* 1994) that allows for recruitment of a subset of coactivators (Giangrande *et al.* 2000) and suppresses the activity of an inhibitory domain contained in both PR-A and PR-B (Giangrande *et al.* 2000). Both isoforms of PR contain all the sequences necessary for proper transcriptional activation. However, in cell-culture studies, PR-A and PR-B display different transactivation properties specific to both cell type and target gene (Meyer *et al.* 1992, Vegeto *et al.* 1993). Although the diverse physiological activities of PR are likely due to the distinct transcription activation properties of each isoform, only a few studies have investigated relevance of the two isoforms in animals. Using mice expressing only one of the isoforms, Conneely & colleagues (Mulac-Jericevic *et al.* 2000, Fernandez-Valdivia *et al.* 2005 and references therein) have described the phenotypic characteristics in the mammary gland and uterine tissue for each isoform-specific genotype. However, the specific roles of PR isoforms in the hypothalamus have not been reported.

Activation of hypothalamic PR in the female influences ovulation and contributes to reproductive behavior. Inhibition of estrogen-induced PR by

antisense oligonucleotides interrupts the gonadotropin-releasing hormone (GnRH) surge that regulates the release of luteinizing hormone (LH) for ovulation (Levine *et al.* 2001). Disruption of estrogen-induced PRs in the ventrolateral portion (vl) of the hypothalamic ventromedial nucleus (VMN) suppresses the PR-mediated reflex lordosis in rodents (Ogawa *et al.* 1994, Apostolakis *et al.* 1996, Pfaff & Schwartz-Giblin 1998). Emanating from the VMN is transneuronal macrocircuitry that ultimately mediates the motor component of lordosis associated with estrogen and Prog (Pfaff & Schwartz-Giblin 1998, Flanagan-Cato *et al.* 2001). The circuitry includes cells of the periaqueductal gray, medullar reticular formation, lumbar ventral horn, and lordosis-producing flank muscles (Pfaff & Schwartz-Giblin 1998) that have been linked serially through retrograde tract-tracing studies (Flanagan-Cato *et al.* 2001). Tactile stimulation that accompanies mounting activates the ascending portion of this circuitry for the initiation of PR-mediated lordosis (Pfaff & Schwartz-Giblin 1998). Although we (Apostolakis *et al.* 1996, 2000, 2002, 2004, 2005) and others (Blaustein 2004) have reported steroid- and/or steroid receptor-independent lordosis induced by select neurotransmitters, neuropeptides, and growth factors, the present study focused on Prog- and PR-mediated sexual behavior. Therefore, we used mice in which PR-A and PR-B were selectively disrupted to analyze the functional role of hypothalamic PR isoforms in female reproduction.

Materials and methods

Animals and treatments

The genotyping protocol and general characteristics of PR isoform-specific knockout (KO)- and null-PRIacZKO mice have been described elsewhere (Mulac-Jericevic *et al.* 2000, Ismail *et al.* 2002). To generate mice deficient in PR-A (PR-AKO), PR-B (PR-BKO), or both isoforms (PRIacZKO), heterozygous females were mated with homozygous males generated from established breeding colonies at Baylor College of Medicine. J P Lydon, B Mulac-Jericevic, and O M Conneely kindly provided the initial heterozygous breeding pairs for PRIacZKO, PR-AKO, and PR-BKO. All mice were housed and maintained under 12 h light:12 h darkness cycle with food and water freely available in accordance with Federal guidelines for humane care using protocols approved by the institutional animal care and research advisory committee.

Experiments were performed on age-matched, wild-type (WT) (C57B/129SV), and mutant knockout mice (males, 60–180 days of age; females, 50–90 days). All females were ovariectomized (ovx) at 40–42 days of age and priming started 7 days later. Before random

assignment to an experimental treatment group, females were primed with estradiol benzoate (EB, 1 µg in 0.05 ml sesame oil, s.c.), then progesterone (100 µg in 0.05 ml sesame oil, s.c.) 44 h later for induction of sexual receptivity. Females were paired with sexually experienced males for behavioral testing, which began 6 h following administration of progesterone. This entire sequence was repeated thrice at 7-day intervals in order to guarantee optimal female receptivity in this hormonal context (Gonzalka & Whalen 1976). During the third exposure, each female-male pair was videotaped (Canon Elura Digital Video Camera, Canon Inc., NY, USA) for 30 min. At a later time, behavior was assessed using frame-by-frame and slow motion analysis by scoring displays of proceptive (acceptance and rejection) and receptive (lordosis) behaviors in females.

Behaviors were defined as follows. Female proceptive acceptance behaviors included female-initiated contact, hopping and darting forward, and immobility while being inspected by male. Proceptive rejection behaviors in response to male approach included those classified as challenge behaviors (en face posturing, standing, kicking, fighting, and/or biting) and mild avoidance behaviors (kicking, jumping upward, and fleeing). Receptivity (positive lordosis) was defined as any dorsiflexion of the vertebral column with head elevation and leg extension in response to male mounting and flank stimulation. The quality of the lordosis reflex response is quantified by an assigned receptivity score (0=no dorsiflexion; 1=some head elevation, back parallel to floor; 2=slight-moderate dorsiflexion, some leg extension from crouch position; 3=immobile full dorsiflexion with head at 45° angle from floor and perineal elevation preceded by flank contact with the male; Pleim *et al.* 1993). Therefore, only scores of 2–3 were considered positive receptivity/lordosis. Stud male behavior was assessed as either inspection (sniffing, grooming of female), aggression (mounting in spite of fighting and/or biting by the female), or sexual (mounting with forepaw stimulation of female flank, intromission, ejaculation). Detailed accounts of the null mutant male aggressive, sexual, and locomotor behaviors are in preparation. Each treatment group was composed of a total of 16–20 animals per gender per genotype. Data were statistically analyzed using two-way ANOVA with the replication for main effects of genotype and steroid treatment and their interaction, followed by *post hoc* one-way ANOVA on steroid treatment when appropriate. Data of non-repeated measurements were analyzed by one-way ANOVAs. *Post hoc* pairwise comparisons were analyzed by Dunnett's Range test. The level of significance was $P \leq 0.05$. Differences in the percentage of animals showing certain behaviors were tested with χ^2 -test.

Experimental behavior and plasma LH testing

Forty-eight hours before experimental testing, female mice were injected with either EB (1 µg in 0.05 ml sesame oil, s.c.) or vehicle (0.05 ml sesame oil, s.c.). Experimental mice were injected according to the relevant experimental protocol. During behavioral testing, each female was allowed to habituate in an empty cage (food and water removed) for 10 min and then the experienced male mouse was placed in the cage. The male was allowed 30 min to mount the female successfully during which time the session was recorded.

All behavioral testing was conducted between 2 and 6 h into the dark cycle (12 h light:12 h darkness cycle, lights off at 1000 h CST) in a quiet room designed for such testing with constant humidity and temperature, controlled lighting, and limited entry and ambient noise levels. Sessions were videotaped and later assessed as described earlier. Individuals evaluating the sessions were blind to the animal genotype (and treatments) and were familiar with testing constraints, rating systems, and normal mouse behaviors. Intertester reliability was conducted to guarantee high correlation between scoring of the observers.

For experiments to assess LH secretion in response to EB and GnRH priming, females were group-housed four to five per cage under 14 h light:10 h darkness cycle with lights on at 0500 h CST. In the behavioral paradigm, 4 weeks of steroid treatment were conducted as described earlier. Females of each genotype ($n=5$ per treatment per genotype) were injected subcutaneously with EB (1 µg) or sesame oil at 0800 h. On the evening of the next day at 2000 h, females were deeply anesthetized and 0.8–1 ml trunk blood was collected following decapitation. Blood was centrifuged and plasma frozen at -70°C for later LH RIA. In a second group of EB-primed females ($n=5$ per treatment per genotype), EB-primed females were administered GnRH (200 ng/kg in saline vehicle, s.c.) at 0900 h. Ten minutes after GnRH or vehicle, one group of females was killed as above and the trunk blood was collected 10 min later. The remaining females were given a second dose of GnRH at 1000 h and euthanized for blood. Blood was stored at -70°C for later RIA.

All injections were prepared immediately before administration unless otherwise stated. Whenever possible, doses were based on published studies for effective concentrations or verified when appropriate. Steroids were dissolved in sesame oil.

Real-time quantitative reverse transcriptase PCR (RT-qPCR)

Brain tissue samples ($n=3$ animals per treatment group per genotype repeated twice) were collected by micropunch technique using anatomical markers (Franklin & Paxinos 1997). Punched-out tissue was

immediately stored in RNALater (Ambion, Austin, TX, USA) at room temperature for 24 h and then at -20°C per manufacturer's recommendations. RNA was extracted using RNeasy Lipid Tissue Mini-kit (Qiagen, Inc.) using the manufacturer's protocol. Total RNA (50 ng) was analyzed by RT-qPCR using a one-step RT-PCR procedure (TaqMan One-Step RT-PCR Master Mix reagents Kit; Applied Biosystems, Foster City, CA, USA) and ABI Prism PE7700 Sequence Analyzer (Applied Biosystems) for target RNAs and 18S RNA expression. All primers and probes were designed using Primer Express software (Applied Biosystems) following Applied Biosystems guidelines. For PRdbd, the forward primer was 5'-GGC TGG CAC TAT GGC GTG CT-3', the reverse primer was 5'-CAT AAA TAG TTA TGC TGC CCT TCC A-3', and the probe was 5'-CTT AAA GAA GAC CTT GCA AGC TCC CAC AGG-3'. For GnRH, the forward primer was 5'-CTG ATG GCC GGC ATT CTA CT-3', the reverse was 5'-A GGG CGC AAC CCA TAG G-3', and the probe was 5'-TTT GGA AGG CTG CTC CAG CCA GC-3'. Primers (300 nM each) and labeled probe (250 nM; 5' label, 6-carboxy-fluorescein; 3' label, 6-carboxy-tetramethyl-rhodamine) were used in 25 µl reaction volume in MicroAmp 96-well plates. Thermal cycling conditions included a RT step for 30 min at 48°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Singleplex quantities were normalized against 18S RNA amplification (primer/probe set by Applied Biosystems), for which input mRNA was diluted 100-fold. Cycle threshold values (Ct) were analyzed using the SDS1.9 software (Applied Biosystems), and relative quantification of mRNA expression was determined using the comparative Ct method (ABI Prism 7700 SDS User Bulletin #2; Applied Biosystems). The slope of log input amount versus δCt for the target genes ranged from -0.04991 to -0.061655 , indicating similar amplification efficiency of each target cDNA and 18S RNA reference. Therefore, the mRNA expression levels were normalized to endogenous 18S RNA and then analyzed relative to vehicle-treated WT tissue. ANOVA ($P\leq 0.05$) was used to statistically compare data.

Single-cell RNA analysis

Using those sections processed for single-cell analysis, individual immunoreactive cells were collected from the vVMN following the laser capture microdissection (PixCell II System; Arcturus Bioscience, Mountain View, CA, USA) technique previously described (Ginsberg & Che 2002). RNA was extracted from laser-captured cells (three individual cells and one pool of 20 individual cells per animal per treatment per genotype) following the manufacturer's protocol for triazol RNA extraction (Invitrogen Life technologies). Single-cell RNA transcripts were amplified following single-cell amplification

protocol pioneered by Eberwine *et al.* (1992) and modified for terminal continuation developed by Ginsberg & Che (2002), Che & Ginsberg (2004). Following amplification and radiolabeling, the RNA was hybridized to custom-made cDNA arrays developed in our lab and that of Ginsberg. The membrane arrays were washed and stored in phosphor screens for 72 h when hybridization intensity of the labeled products was detected by phosphor imaging and scanner (ImageStorm Scan, Molecular Dynamics, Piscataway, NJ, USA) and ImageQuant Software (Molecular Dynamics). cDNA for pScript was used as background control and its hybridization signal intensity was subtracted from the intensity value of each individual labeled cDNA before statistical analysis. Following this, a ratio was generated between the expression of the hybridized RNA and the total hybridization signal intensity of the membrane array, thus allowing for a comparison of relative changes of mRNA using ANOVA. Individual comparisons were also analyzed using a *post hoc* Newman–Keuls test. Data are plotted as the logarithmic transformation of the above ratio and error bars denote s.e.m. Significance was $P \leq 0.05$. Between all arrays, more than 570 genes specific to steroid receptor activation and neurological processes were examined for RNA expression. Since most of these genes are beyond the scope of this manuscript, only a select number of those genes most relevant to lordosis are presented herein. A comprehensive presentation of all genes is in preparation. Each cDNA on the custom cDNA arrays was verified by restriction digestion and sequence analysis.

Immunohistochemistry (IHC) for protein and single-cell RNA analyses

IHC for single-cell RNA detection

To identify the cells of interest in the vVMN, brain sections were processed for IHC using antibodies to PR (on WT, PR-AKO, PR-BKO tissue) and lacZ (PRlacZKO tissue). Brain tissue samples of deeply anesthetized mice ($n=3$ animals per treatment group per genotype) were collected following transcardiac perfusion with sterile PBS followed by cold 70% ethanol buffered with 150 mM sodium chloride. Tissue was post-fixed in 70% ethanol and 150 mM sodium chloride for 1 h at 4 °C, then washed and cryoprotected in 30% sucrose and PBS with RNase inhibitor (Invitrogen Life Technologies) overnight at 4 °C. Tissue was then washed and embedded in tissue freezing medium (Electron Microscopy, Fort Washington, PA, USA) and placed at -80 °C for a minimum of 24 h before sectioning. Tissue was cryosectioned into 5–7 μm sections at -20 °C. Immunostaining was employed using non-specific blocking with 3% goat serum and RNase inhibitor in PBS and sections were incubated in a primary antibody against PR (DakoCytomation, Inc.,

Carpinteria, CA, USA; 1:100 in PBS with 3% goat serum, 1% BSA, 0.5% triton, and RNase inhibitor) or galactosidase (Promega; 1:1000 with 10% goat serum). Twenty-four hours later, tissue was incubated for 1 h at room temperature in a biotinylated anti-rabbit (1:500, Vector-stain Elite Kit; Vector, Burlingame, CA, USA for PR) or anti-mouse (1:500, Vector Elite Kit, for galactosidase). Positive vVMN cells from tissue sections covering the region from -1.84 to -1.94 mm caudal to bregma (Franklin & Paxinos 1997) were visualized using Vector ABC and DAB Kits per the manufacturer's recommendations (Vector Laboratories). Slides were stored in PBS and RNase inhibitor at 4 °C until laser capture dissection.

IHC for protein detection

A modified version of the above IHC protocol was used for protein detection and has been previously described (Apostolakis *et al.* 2000). Briefly, paraformaldehyde (4% in cold PBS) was substituted for ethanol solution and RNase inhibitor was deleted. Positive cells were visualized using Vector ABC and DAB Kits (Vector) per the manufacturer's recommendations and cover-slipped after graded dehydration in EtOH and xylene.

Analysis of PR immunoreactivity (ir) was computer-assisted using an Olympus BM60 microscope (Leeds Instruments, Dallas, TX, USA), fitted with a Macintosh G4 computer and interfaced via Spot Digital Program. Cell counting was automated with Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA). The microscope was adjusted for Kohler illumination and focused on a black circle that had been affixed so that the black circles produced gray levels of 254 U and the blank portions of the slide produced gray levels of approximately 5 U. Cells were considered immunoreactive if they had >10 pixels but <200 pixels in area, and the pixel optical density exceeded an average optical density of the surrounding tissue by a predetermined number of SDs. Since the difference in optic density between the irPR and the surrounding tissue varied between areas, the number of SDs varied according to the area that was analyzed. However, the same criterion was used for every section and VMN region analyzed. The irPR were quantified in the lateral VMN, as illustrated previously (Moffatt *et al.* 1998, Flanagan *et al.* 2001). This area was chosen because it is known to contain the majority of neurons containing EB-induced PRs that are serially linked through projections of the periaqueductal gray, vestibular complex, and reticular formation to the motoneurons of the lumbar muscles that produce the lordosis reflex (Pfaff & Schwartz-Giblin 1998, Flanagan *et al.* 2001). Two-way ANOVA with replication for main effects of genotype and steroid treatment and their interaction was used for data analysis, followed by *post hoc* one-way ANOVA on steroid treatment when appropriate. The level of significance was $P \leq 0.05$.

Plasma LH RIA

The mouse LH IRMA RIA was kindly performed in the Ligand Assay and Analysis Core laboratory under the direction of D Haisenleder at The University of Virginia, using reagents provided by the National Hormone and Pituitary Program. The RIA standards were NIDDK RP-3. The sensitivity of LH was 0.08 ng/tube. Intraassay coefficients of variance for LH were 4.9 and 8.3%. LH is presented as mean \pm s.e.m. All samples from each experiment were assayed in a single RIA assay. Groups were compared using two-way ANOVA with Dunnett's Range tests and $P \leq 0.05$ was considered significant.

Results

WT stud male behaviors toward isoform-specific females

Female steroid milieu is thought to play a role in sexual recognition by males. Three types of behavior were used to assess male sexual recognition of females with different genotypes. There were significant female genotype differences and interactions between female genotype and treatment in all three measures, i.e. frequency of male aggressive, investigative, and sexual behaviors (two-way ANOVA, $P \leq 0.05$).

Aggressive bouts (mounting in spite of fighting and/or biting by the female)

Post hoc comparisons for main effect of genotype revealed a significant increase in male aggression only toward PRLacZKO and PR-BKO females treated with EB+Prog (Fig. 1a, $P \leq 0.05$) but not toward WT regardless of steroid treatment and PR-AKO females treated with EB+Prog ($P \geq 0.05$).

Investigative bouts (sniffing and grooming)

Again, *post hoc* comparisons detected significant female genotype differences within the EB+Prog-treated group (Fig. 1b, $P \leq 0.05$) in that males investigated PRLacZKO females significantly less than other genotypes ($P \leq 0.05$) and at a frequency similar to that of other treatment groups ($P \geq 0.05$). The exception was that EB-primed PR-BKO females elicited a similar increase in male investigative behaviors as EB+Prog-treated WT, PR-AKO, and PR-BKO females (Fig. 1b, $P \geq 0.05$).

Sexual bouts (mounting, intromission, ejaculation)

Steroid milieu and disruption of female PR isoforms were correlated with male sexual behavior. For EB+Prog females, stud males displayed a substantial (and comparable) increase of sexual behaviors toward WT and PR-BKO females (Fig. 1c, $P \leq 0.05$). For PR-AKO females, EB+Prog treatment was correlated with

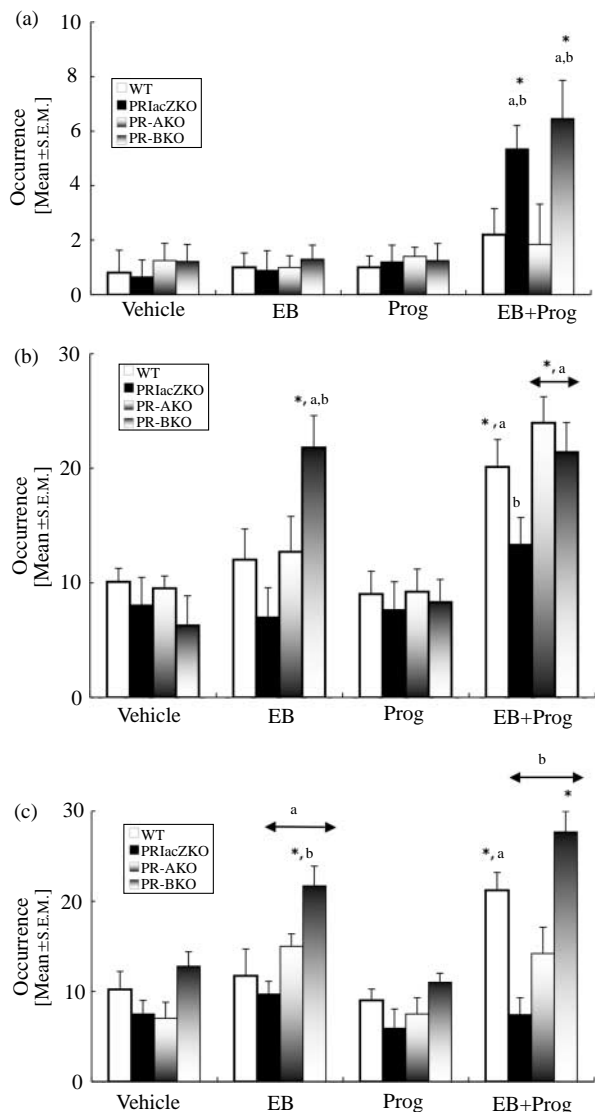


Figure 1 Effects of PR isoforms in ovx female mice on male interactions, including (a) aggressive, (b) investigative, and (c) sexual behaviors. Data were analyzed by two-way ANOVA followed by one-way ANOVA and Dunnett's Range tests. *Significance was $P \leq 0.05$ for vehicle-treated WT versus treated mice, ^avehicle-treated mice of same genotype versus treated mice and ^bWT versus other genotypes within treatment group.

increased male sexual behavior compared with vehicle-treated PR-AKO (Fig. 1c, $P \leq 0.05$), but not WT and PR-BKO females ($P \geq 0.05$). Males exhibited the most sexual behavior toward EB-primed PR-BKO females (Fig. 1c, $P \leq 0.05$). Steroid treatment failed to elicit male sexual interest in PRLacZKO females (Fig. 1c, $P \geq 0.05$).

Altogether, the data support the hypothesis that male recognition of receptive females is mediated by the interaction of female genomics with EB+Prog treatment. In other words, WT males displayed the greatest interest and sexual drive toward those females expressing PR-A

(WT, PR-BKO) who were under a steroid milieu that favors the facilitation of receptivity. Therefore, WT males favor PR-A-expressing females with their 'courtship' behaviors.

RNA and protein expression in the female brain

Rodent female receptivity is exquisitely dependent on EB-induced expression of PR in the VMN (Pfaff *et al.* 1988), possibly through a half-site estrogen response element near the ATG start site (Kastner *et al.* 1990). In cell culture, both PR-A and PR-B are estrogen inducible (Kraus 1993).

PR expression in punched-out VMN

Primers and TaqMan probe designed to detect a common nucleotide sequence (PRdbd) within the DNA-binding domain of the mouse PR was retained within the mice studied (Lydon *et al.* 1995, Mulac-Jericevic *et al.* 2000, Fernandez-Valdivia *et al.* 2005) and used to verify the induction of each PR isoform in the punched-out VMNs. EB induced transcription of PR mRNA in WT, PR-AKO, and PR-BKO female VMNs (Fig. 2a, $P \leq 0.05$). PRdbd expression was comparable within each treatment group (Fig. 2a, $P \geq 0.05$). As expected, PR transcription was

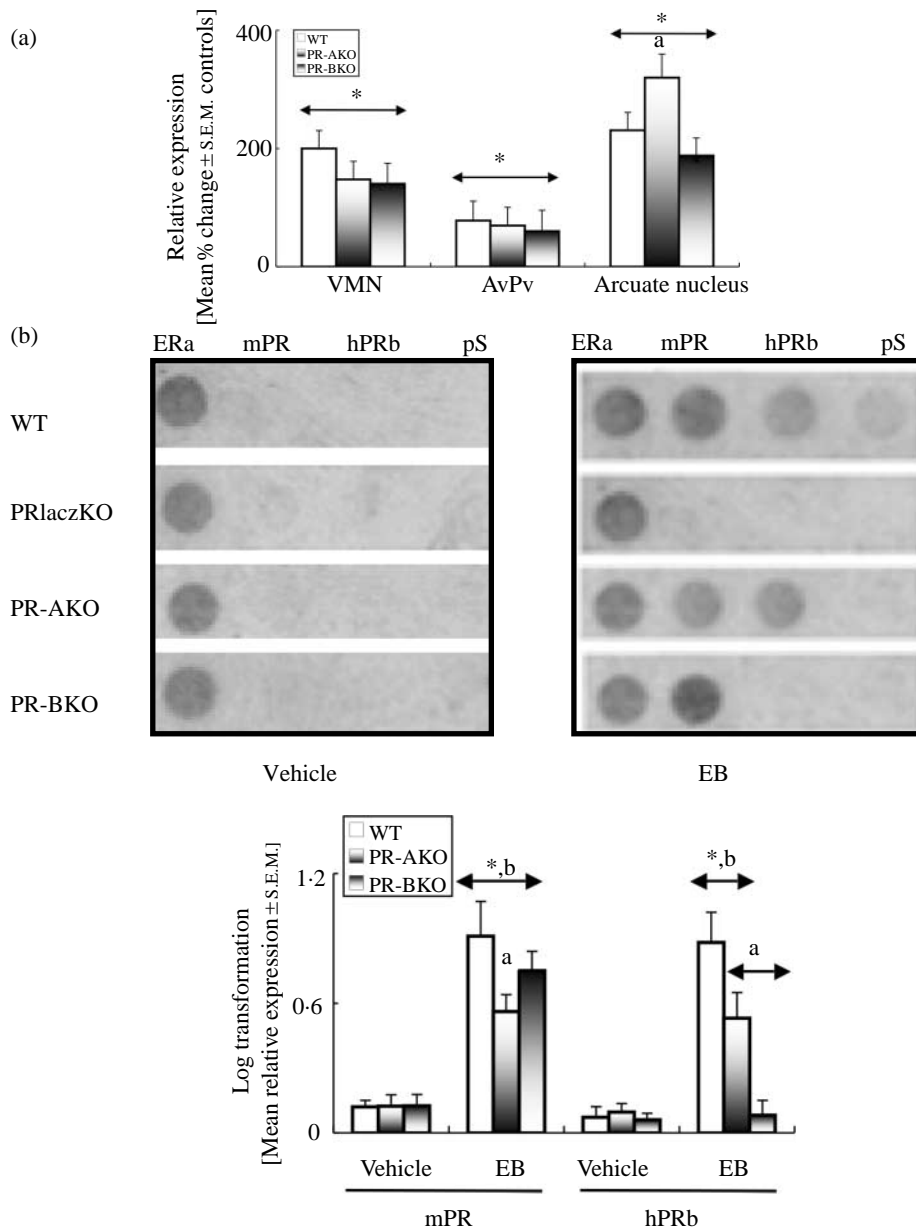


Figure 2 (continued)

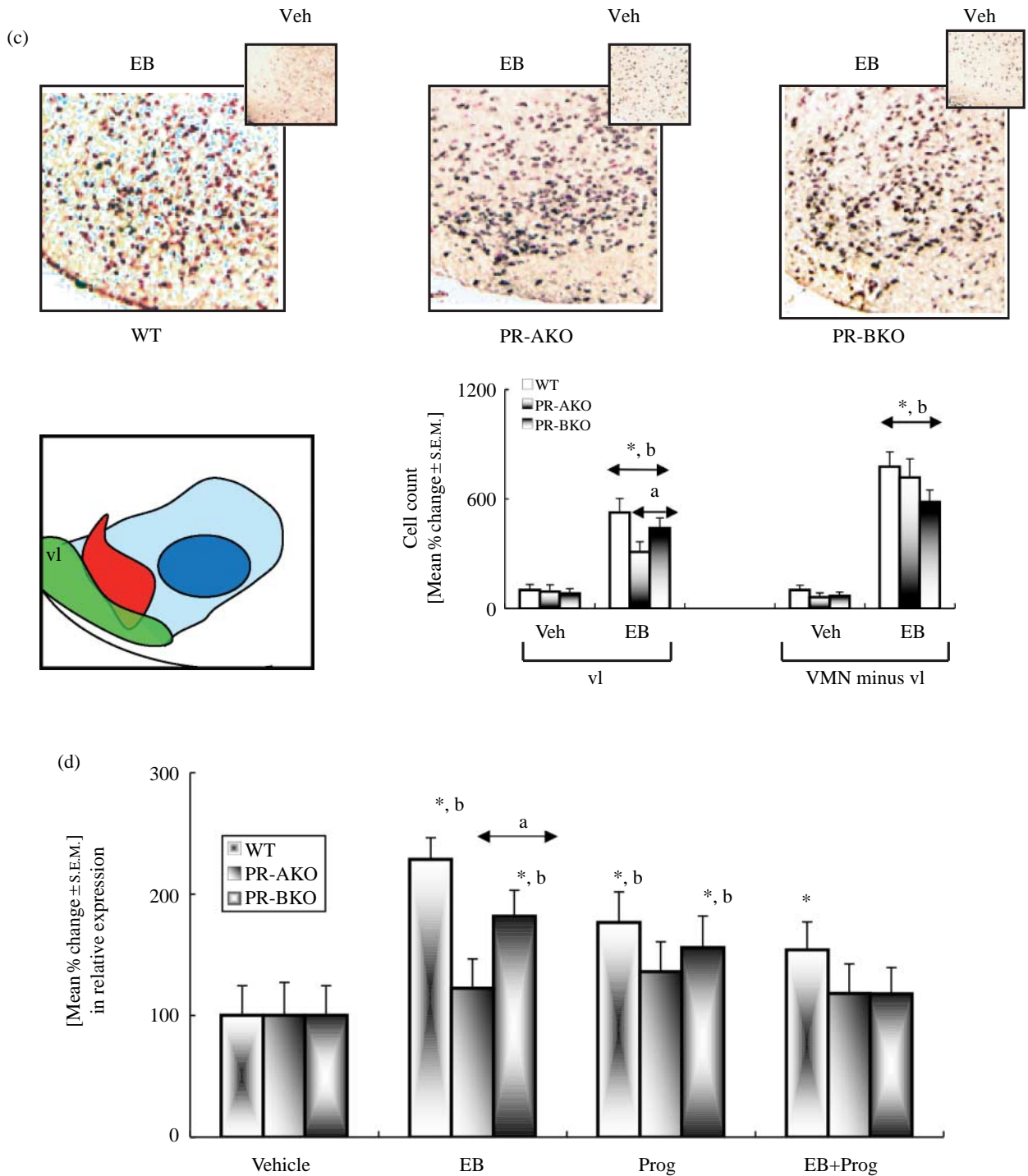


Figure 2 (continued)

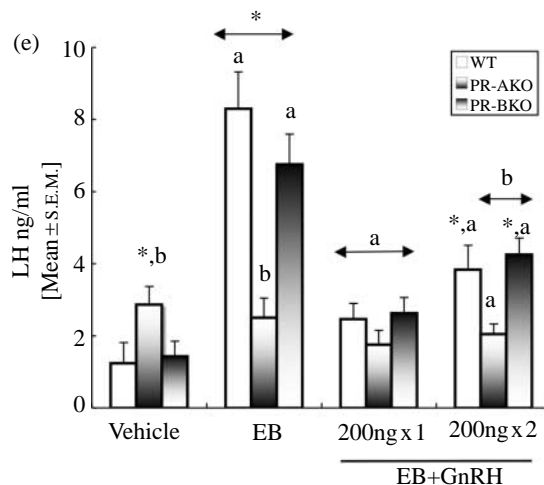


Figure 2 Effects of PR isoforms on RNA and protein expression in the vVMN of ovx mice assessed by real-time RT-PCR (a and d), single-cell mRNA profiling (b), representative blots from individual female vVMN neurons and graphic of log transformed ratio between hybridization signal of the particular gene versus background hybridization signal for blot, immunoreactive PR in the vVMN (c), and RIA for the percentage changes in plasma LH (e). In c, the green vVMN region is oxytocin and ER α immunoreactive and contains the majority of VMN neurons serially connected to lumbar muscles that produce lordosis (Flanagan-Cato *et al.* 2001). The neurons in the blue region display EB-induced spines, whilst the red region contains dense clusters of ER α -containing neurons. * $P \leq 0.05$ for vehicle-treated WT versus treated mice, ^avehicle-treated mice of same genotype versus treated mice, and ^bWT versus other genotypes within treatment group. Veh, vehicle.

minimal with Prog only treatment and downregulated by Prog in EB-primed females (data not shown). Hence, EB priming regulated *in vivo* transcriptional levels of both PR-A and PR-B isoforms in the female VMN.

PR expression in punched-out anteroventral periventricular nucleus (AvPv). Previous studies have demonstrated the importance of EB-induced AvPv PR for the GnRH surge and for receptive behavior (Chappell & Levine 2000). Compared with vehicle-treated AvPvs of the same genotype or WT, EB induced PR expression in the AvPv (Fig. 2b, $P \geq 0.05$). As in the VMN, PR mRNA was minimal in Prog only and EB + Prog-treated AvPvs of WT and PR-AKO females (data not shown). Therefore, EB also induced PR isoform expression in the AvPv.

PR expression in punched-out arcuate nucleus

Long hypothalamic fibers from the arcuate nucleus extend to surround and innervate the VMN (Risold *et al.* 1994). Similar to that in punched-out VMN and AvPv, EB alone (Fig. 2a, $P \leq 0.05$) but not Prog only or EB + Prog treatments ($P \geq 0.05$) stimulated PR mRNA in the arcuate nucleus of all PR-expressing genotypes. Moreover, PR expression in EB-primed PR-AKO arcuate nucleus exceeded that detected in primed WT and PR-BKO arcuate nuclei (Fig. 2a, $P \leq 0.05$).

PR expression in individual vVMN cells of PR-lineage

Single-cell RNA analysis (Eberwine *et al.* 1992, Ginsberg *et al.* 2002) of individual neurons with PR lineage was used to assess relative expression and determine colocalization with other critical transcripts in the region of the female vVMN that regulates receptivity. Representative mini-expression profiles of single neurons are shown in Fig. 2b.

RNAs for ER α , PR (A and/or B isoforms, mPR), and/or PR-B (hPRb) were colocalized in individual vVMN neurons from EB-primed WT, PR-AKO, and PR-BKO (Fig. 2b). Although not shown, oxytocin and preproenkephalin were also coexpressed in these individual neurons. As expected, the mPR primers and probe detected PR mRNA in both PR-AKO and PR-BKO neurons (Fig. 2b, blots). The specificity of the hPRa primers and probes was evident, as PR-A was seen in PR-BKO neurons but not in PR-AKO neurons. PR RNA was not detected in vVMN PRlacZKO neurons and no RNA was visible for the negative hybridization controls (pS, Fig. 2b). A significant difference in PR expression was detected from all EB-primed neurons compared with those vehicle-treated WT and same genotype neurons (Fig. 2b, graph, $P \leq 0.05$). Of particular interest, there was a small but significant difference in total mPR expression within individual neurons from EB-primed PR-BKO females compared with that from PR-AKOs (Fig. 2b, $P \leq 0.05$) but not WT

($P \geq 0.05$). In EB-primed WT neurons, hPRb expression was $41 \pm 4\%$ of total mPR. Since single-cell RNA 'fingerprinting' is sensitive to relative changes in expression, these findings raise the possibility that within individual neurons in EB-primed vVMN, the ratio of PR-A:PR-B RNA may favor PR-A.

PR protein expression in the female VMN

IHC and cell counting were used to assess protein expression because protein yield from the rostral subdivision of vVMN is below the level of sensitivity for western blot or RIA. An antibody that recognizes both PR-A and PR-B was used (Mulac-Jericevic *et al.* 2000, Fernandez-Valdivia *et al.* 2005). Consistent with RNA data, EB priming induced immunoreactive PR protein in WT, PR-AKO, and PR-BKO VMNs (Fig. 2c, top panels). Within the VMN from PR-AKO females, a cluster of prominent staining extended from the vl (a rostral region that contains neurons identified with lumbar epaxial muscles in retrograde tract tracing studies (Fig. 2c, upper two-thirds of green schematic in lower left schematic)) to the region (dark blue in lower left schematic), where EB is known to induce synaptic spines (Commons *et al.* 2000, Daniels & Flanagan-Cato 2000, Flanagan-Cato *et al.* 2001). In PR-BKO VMN, prominent staining also was detected in the rostral vVMN, whereas staining in the VMN where synaptic spines develop with EB priming was less dense than that observed in PR-AKO. There was a statistical difference in isoform protein expression in the vl following EB priming (Fig. 2c, graphic, $P \geq 0.05$) with the expression in PR-BKO vl exceeding that in PR-AKO vl. In the VMN minus vl, PR expression in the EB-primed PR-AKO female exceeded that in PR-BKO (Fig. 2c, graphic, $P \leq 0.05$). These findings are consistent with the single-cell fingerprints and suggest differential function of the two PR isoforms within subdivisions of the VMN.

GnRH expression in the punched-out medial preoptic area (mPOA)

The synthesis of GnRH in the mPOA and its secretion are critical for behavioral receptivity. Further, blockade of PR in the AvPv suppresses GnRH in the mPOA and disrupts ovulation (Chappell & Levine 2000). Constitutive GnRH mRNA expression in all female mPOAs was statistically comparable regardless of genotype (Fig. 2d, $P \geq 0.05$). Both EB priming and Prog only significantly enhanced GnRH transcription in the punched-out mPOAs from WT and PR-BKO females but not PR-AKO (Fig. 2d, $P \leq 0.05$ vs $P \geq 0.05$). EB + Prog was associated with a small but significant increase in GnRH only in WT mPOA (Fig. 2d, $P \leq 0.05$). Taken

together, steroids alone significantly enhanced mPOA GnRH transcription levels in WT and PR-BKO females and significantly downregulated it when given together.

Plasma LH in EB-primed ovx females

To ascertain the efficiency of GnRH to induce pituitary LH secretion, plasma LH concentrations were measured following EB priming. Basal levels of LH in vehicle-primed PR-AKO females exceeded that of WT and PR-BKO (Fig. 2e, $P \leq 0.05$). As expected, EB priming induced higher LH levels in WT and PR-BKO females compared with vehicle-primed and EB-primed females regardless of genotype (Fig. 2e, $P \leq 0.05$). Plasma LH was higher in EB-primed PR-AKO females compared with vehicle WT (Fig. 2e, $P \leq 0.05$) but not with vehicle PR-AKO females ($P \geq 0.05$). EB decreased LH levels in PRLacZKO mice compared with vehicle WT and vehicle PRLacZKO (data not shown). Therefore, EB induced an LH surge in the presence of PR-A, but not PR-B.

When female mice are given repeated pulses of GnRH following EB priming, a PR-dependent self-priming mechanism is thought to generate a net increase in the magnitude of LH responses (Levine *et al.* 2001). To determine the presence of GnRH self-priming, a dose of GnRH was given twice with a 1-h interval between doses to EB-primed mice. The first dose of GnRH induced a small, submaximal LH increase in WT and PR-BKO females compared with vehicle WT and PR-BKO mice (Fig. 2e, $P \leq 0.05$), whilst no change ($P \geq 0.05$) and a small decrease ($P \leq 0.05$) in LH were detected in PR-AKO females compared with vehicle WT and PR-AKO mice respectively. LH also increased in PRLacZKO females with the first GnRH dose (data not shown). Following a second dose of GnRH, LH concentrations exceeded those detected after the first dose in WT and PR-BKO (Fig. 2e, $P \leq 0.05$), but not PR-AKO mice (Fig. 2e, $P \geq 0.05$). PRLacZKO females also failed to have changes in LH levels following the second GnRH pulse (data not shown). Hence, self-priming was dependent upon the presence of PR-A.

Steroid-dependent female reproductive behavior

As a reflex in rodents, lordosis is exquisitely dependent upon ER α -mediated synthesis of PR in the VMN (Ogawa *et al.* 1998, Apostolakis *et al.* 2000). Hence, female receptivity is an effective bioassay for studying cellular and molecular mechanisms regulating ovarian steroid receptors *in vivo*.

Receptivity

Receptivity was measured by lordosis quotient (LQ, percent lordosis per total mounts $\times 100$) and consists of

(1) immobile dorsiflexion of the female vertebral column with head at a 45° angle from floor and (2) perineal elevation followed by flank contact with the male. As expected for negative-treatment controls (vehicle-, EB- and Prog-only), male mounting elicited little (if any) response from ovx females regardless of

genotype (Fig. 3a, $P \geq 0.05$). Also as expected for positive-(WT) and negative-(PrlacZKO) genotype controls, EB in combination with Prog-facilitated positive receptivity in WT females (Fig. 3a, $P \leq 0.05$) and little if any receptivity in PrlacZKO females ($P \geq 0.05$). In the experimental animals scored for both dorsiflexion and

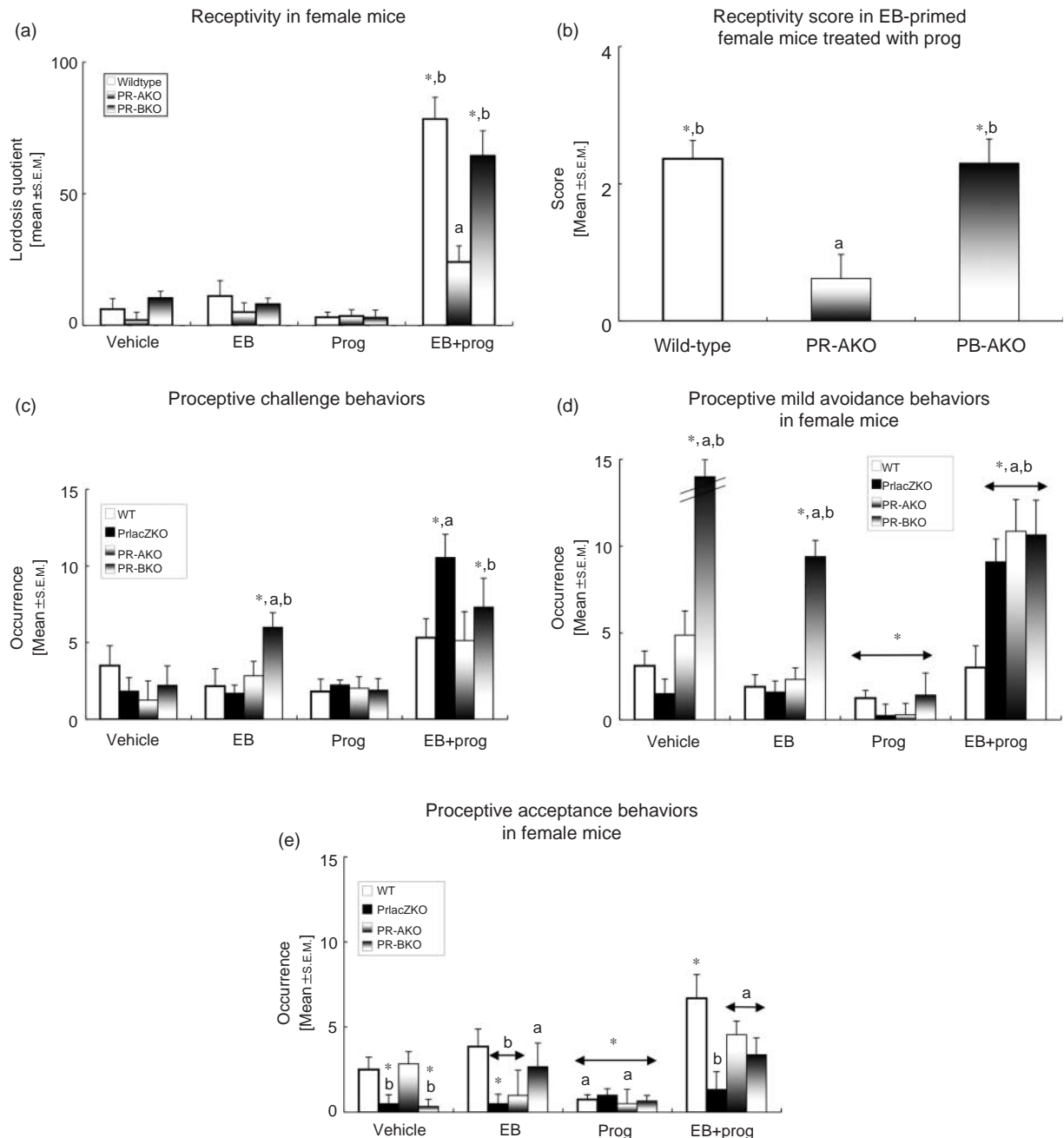


Figure 3 Effect of PR isoform on female reproductive behaviors assessed by mean (\pm s.e.m.) lordosis quotient (a), receptivity score (b), and incidences of proceptive behaviors (c, challenge behaviors; d, mild avoidance behaviors; e, acceptance behaviors) in ovx mice. * $P \leq 0.05$ for vehicle-treated WT versus treated mice, ^avehicle-treated mice of same genotype versus treated mice, and ^bWT versus other genotypes within treatment group.

perineal elevation, EB-primed PR-AKO females failed to significantly exhibit positive receptivity in response to Prog (Fig. 3a, $P \geq 0.05$). In contrast, EB+Prog-treated PR-BKO females displayed positive lordosis ($P \leq 0.05$). These findings support the hypothesis that PR-A mediates exhibition of full lordosis posturing.

Receptivity score

The quality of the lordosis reflex response was quantified using an assigned receptivity score (0=no dorsiflexion; 1=some head elevation, some foreleg extension; 2=slight moderate dorsiflexion or back parallel to the floor with attempts at or some hindleg extension from crouch position; 3=full dorsiflexion; Pleim *et al.* 1993). In response to all negative control treatments, receptivity scores were <1 (data not shown). In contrast, EB-primed WT and PR-BKO females treated with Prog often scored 2 or greater, whereas in EB+Prog treatment of PRLacZKO females predominately scored 0 (Fig. 3b, $P \leq 0.05$). The positive response of WT and PR-BKO mice appeared immediately after male mounting, flank stimulation, and modest male thrusting. PR-AKO females receiving EB+Prog were more difficult to score because they often displayed head elevation and, in a modest number of females, delayed foreleg extension after prolonged male mounting, intromission, and persistent thrusting (Fig. 3b). This posturing tended to flatten the vertebral column and often did not produce dorsiflexion. Although the data support the hypothesis that PR-A isoform in the VMN mediates reflex sex behavior in female mice, the absence of PR-A expression does not constrain female mice from attaining a posture conducive to male copulation. Indeed, extensive male tactile stimulation of the flanks, perineum and, possibly, vagina ultimately led to a posture that is reminiscent of that observed in female rats who have experienced repeated, extensive tactile stimulation (Pfaff & Schwartz-Giblin 1998).

Female proceptive behaviors (challenge, mild avoidance, acceptance)

Coincident with receptivity is a period of time when females exhibit a series of 'courtship' behaviors that foster behavioral interactions with the males and facilitate the electrophysiological properties of the neurons in the female hypothalamus. It is likely that these behaviors, while not reflex, are due to hormonal modulation of the serial neurocircuitry via ER (Pfaff & Schwartz-Giblin 1998), inducible PR (Monks *et al.* 2001), and at least SRC-1 (Monks *et al.* 2003). To verify this, the incidence of proceptive behaviors by the females was quantified.

Proceptive challenge behaviors (en face posturing, standing, fighting, and/or biting)

Although constitutive levels of challenge behaviors were statistically similar between the genotypes, PR-AKO females tended to respond less frequently with challenge behaviors (Fig. 3c, $P \geq 0.074$). WT and PR-AKO females did not display a change in the incidence of challenge behaviors in response to steroids (Fig. 3c, $P \geq 0.05$). EB priming resulted in PR-BKO females exhibiting standing and en face posturing, behaviors that persisted after Prog was administered (Fig. 3c, $P \leq 0.05$). PRLacZKO females exhibited standing and fighting with EB+Prog treatment (Fig. 3c, $P \leq 0.05$). For EB+Prog-treated females, fighting was predominant in PRLacZKO mice and biting in PR-BKO females. Therefore, EB alone and in combination with Prog facilitated the appearance and the type of challenge behaviors exhibited by PRLacZKO and PR-BKO, whereas WT and PR-AKO female mice exhibited little if any change in the incidence of challenge behavior in response to steroids.

Proceptive mild avoidance behaviors (fleeing, jumping, and kicking)

Female genotype accounted for a marked difference in the incidence of fleeing and jumping behaviors. Constitutively, PR-BKO females exhibited a significantly high incidence of jumping, a behavior that was constrained by EB alone and in combination with Prog (Fig. 3d, $P \leq 0.05$). Prog alone suppressed kicking below constitutive levels (Fig. 3d, $P \leq 0.05$). Following EB+Prog treatment, PRLacZKO and PR-AKO females exhibited increased kicking bouts (Fig. 3c, $P \leq 0.05$) to levels comparable with PR-BKO ($P \geq 0.05$). Therefore, the steroid milieu that facilitated reproductive behavior (EB+Prog) modulated the exhibition of mild avoidance behaviors in all mutant females.

Proceptive acceptance behaviors (initiating contact with inattentive, hopping and darting, pausing for attentive male)

Vehicle-treated WT and PR-AKO females displayed comparable frequencies of acceptance behaviors (Fig. 3e, $P \geq 0.05$), whilst PRLacZKO and PR-BKO females had fewer bouts ($P \leq 0.05$). Likewise, PRLacZKO females failed to display acceptance behaviors regardless of steroid treatment regimen, whereas these behaviors were suppressed in WT and PR-AKO females receiving Prog only (Fig. 3e, $P \leq 0.05$). Of interest because it enhances neuronal facilitation in spinal neurons, hopping and darting was displayed only by WT females and then only after EB+Prog treatment (Fig. 3e, $P \leq 0.05$). PR-AKO treated with EB+Prog initiated

contact more than other mice (data not shown, $P \leq 0.05$). Unlike the rat, ear wiggling was not observed in any mouse. As before, genotype determined the type of acceptance behaviors, whereas steroid treatments influenced the occurrence and type of acceptance behaviors.

Taken together, there was a pattern of female proceptive behaviors associated with genotype and steroid milieu. Under the steroid regimen associated with positive lordosis, null isoform females more often displayed rejection behaviors, whereas only PRLacZKO females failed to exhibit any acceptance behaviors. These findings support the hypothesis that PR isoforms differentially play a role in female proceptive behaviors.

Discussion

In the present study of female mice with mutant PR isoform expression, several essential elements that regulate female receptivity were explored. In ovx females primed with EB, PR-A RNA and protein expression dominated in individual neurons of the vVMN, a small region that contains the greatest number of EB-induced PR-expressing neurons serially connected to motoneurons of lumbar muscles mediating lordosis. PR-B predominated regions where neurons display estrogen-induced spines, allowing for its dominance in punched-out vVMN tissue. In addition, the levels of GnRH mRNA in the mPOA were greater in those EB-primed females expressing PR-A. Plasma LH concentrations were induced by EB priming and self-priming by two GnRH pulses in PR-A-expressing females. Proceptive behaviors that attract male interaction and foster lordosis were displayed more often in EB-primed PR-A-expressing females given Prog. Hence, it was not surprising that those females-expressing PR-A (PR-BKO) exhibited lordosis and also had greater receptivity scores than the PR-A-expressing counterparts.

Mating behavior is a symphony of interactions between males and females predominantly influenced by female steroids and pheromones, some of which are Prog metabolites (Schwende *et al.* 1984, Pling *et al.* 2001, Poling *et al.* 2001, Stacey *et al.* 2003). In turn, activation of the male vomeronasal-accessory olfactory system generates electrophysiological changes in the forebrain circuitry for precopulatory behaviors (Pfaff 1999, Holy *et al.* 2000, Luo & Katz 2004). In the present study, when the steroid milieu of the female favored receptivity (EB+Prog), WT males investigated females of all three genotypes, with PRLacZKO being the least investigated. Fewer aggressive bouts were exhibited toward PR-AKO females, whereas more sexual bouts occurred toward PR-BKO females. These findings are consistent with the notion that Prog components and PR play a role in the formation of female pheromones that evoke distinctive patterns of behavior in males.

The specific contribution of each PR isoform is dependent upon the ratio of individual isoforms in a given cell. The two isoforms are frequently expressed in the same reproductive cells at varying levels depending on development, hormonal status, and with cancer (Mangal *et al.* 1997, Graham *et al.* 1999, Graham & Clarke 2002). When expressed in equimolar ratios in a given cultured cell, PR-A and PR-B are thought to dimerize and bind DNA-response elements as either A:A or B:B homodimers or A:B heterodimers, each with different transactional properties depending on the absence or presence of N-terminal domain of the PR-B gene (Sartorius *et al.* 1994, Wen *et al.* 1994). In mammary tissues, overall predominant expression of PR-A is an early event in ductal carcinoma and invasive lesions (Mote *et al.* 2002), whereas overexpression of PR-B indicates poorer outcome with chemotherapy (Graham *et al.* 2005). Indeed, overexpression of PR-A is associated with the acquisition of Prog responsiveness in a number of genes that are normally not Prog targets (Graham *et al.* 2005). In the uterus, PR-B is low during low estrogen states and enhanced with estrogen levels during the follicular phase (Mangal *et al.* 1997). Disruption of the ratio of PR-A:PR-B is correlated with Prog resistance in endometriosis (Attia *et al.* 2000) and malignant endometrial cancer (Arnett-Mansfield *et al.* 2004). In the present study, PR-A and PR-B mRNA were colocalized in individual WT neurons of the most lateral vVMN with EB induction. In the isoform-specific mice treated with EB, the ratio of PR-A:PR-B mRNA favored PR-A in the area of the vVMN where neurons have transsynaptic linkage to lordosis-producing lumbar muscles (Flanagan-Cato *et al.* 2001). These findings were confirmed for protein expression in the isoform-specific mice but not WT mice due to technical limitations. In contrast to individual neurons, the ratio shifted toward favoring PR-B in punched-out VMN tissue, a finding consistent with that in rat MBH (Kato *et al.* 1994, Guerra-Araiza *et al.* 2000, 2003). The present study does not determine whether PR-A acts as an inhibitor of PR-B activity by forming heterodimers with PR-B in WT cells (Graham & Clarke 2002). Nevertheless, the present data provide evidence for differing PR-A:PR-B ratios in the two subdivisions of the VMN following EB priming and are consistent with PR-A mediation of positive lordosis.

Lordosis is controlled by a transsynaptic network of neurons that begins in a specific subdivision of the vVMN and transverses the spinal column to ultimately innervate the lumbar epaxial muscles (Flanagan-Cato *et al.* 2001). Relatively small in number, these neurons are located in the most vl portion of the VMN and, after EB priming, can be defined by oxytocin fiber ir and coexpression of PR with ER α . In experiments of EB-primed single vVMN neurons, both PR isoforms were colocalized with ER α and oxytocin, thus

confirming that the most relevant population of neurons for our behavioral studies was selected for molecular fingerprinting. If the present findings for synthesis of vVMN PR-A and PR-B in the PR-isoform-specific mice are proportionally similar in WT vVMN, then PR-A is the predominant isoform in this small region. In support of this, morphological changes observed in hypothalamic cells (Pfaff & Schwartz-Giblin 1998) are similar to those cytoskeletal changes associated with PR-A predominance in other tissue (McGowan *et al.* 2003). Importantly, the present behavioral findings for lordosis show that those females expressing PR-A display Prog-induced lordosis behavior, whereas PR-B-expressing females exhibit slow, incomplete dorsiflexion following persistent tactile stimulation of the flanks and vagina. These findings substantiate the role of vVMN PR-A in Prog-facilitated lordosis and demonstrate the value of single-cell fingerprinting.

In VMN subdivision containing densely packed clusters of neurons known to display EB-induced spines (Frankfurt *et al.* 1990, Calizo & Flanagan-Cato 2000), PR-B predominance was detected for both mRNA and protein. Since individual neurons are endowed with the capacity to independently control synaptic strength through local synthesis of proteins (Wiersma-Meems *et al.* 2005, Sutton & Schuman 2005), PR-B predominance in this region may suggest that it has a role in modifying local neuronal activity. Stimulus-driven activity of the PR-B-dominant neurons could be recruited to sensitize and/or optimize downstream neuron activity for more robust muscle function and extension of the time females remain in dorsiflexion. Such a role may be critical under less than optimal steroid conditions when an optimized reflex is required. Therefore, one could speculate that PR-B is fine-tuning the activation of receptivity by extending the sphere of PR influence to other VMN subdivisions. It will be interesting to determine the molecular fingerprints of these adjacent neurons. Within individual neurons, PR-B also may be regulating the downregulation of ER α in the VMN, an effect that, *in vitro*, is mediated by both PR-A and PR-B in a ligand and cell-specific manner (DonCarlos *et al.* 1995, Katzenellenbogen 2000). Previous protein and mRNA studies have shown such a role for PR in other reproductive tissue (Ismail *et al.* 2002).

Prog receptors in the AvPv, a region that sends projections to the mPOA (Pfaff & Schwartz-Giblin 1998, Simerly *et al.* 1996), play an indispensable part in the timing mechanism for the LH surge (Chappell *et al.* 1999). As such, AvPv PRs also influence female sex behavior (Chappell & Levine 2000, Levine *et al.* 2001, Hahn & Coen 2006). In the present study, EB priming enhanced PR in the AvPv, GnRH expression in the mPOA, and plasma LH concentrations in WT and PR-BKO females. Likewise, two pulses of GnRH resulted in a net increase in plasma LH in WT and PR-BKO,

suggesting PR-A participates in GnRH self-priming and LH surge generation. In contrast, there was an absence of self-priming in PR-B-expressing (and PRLacZKO) mice given two GnRH doses since there was no additional increase in LH. Basal levels of plasma LH and mPOA GnRH mRNA were greatest in PR-B-expressing (and PRLacZKO) mice, possible due to larger gonadotropin increase after ovx or retention of GnRH pools between weekly EB priming that preceded experimentation. These findings are consistent with previous data in WT and PRKO mice (Chappell *et al.* 1999). Altogether, PR-A is likely the relevant PR required for transmission of EB induction of gonadotropin surges.

Finally, female proceptive (aka 'courtship') behaviors are associated with hormonal status and facilitate neuronal electrophysiological properties in order to prepare the female for successful lordosis (Pfaff & Schwartz-Giblin 1998). For example, EB induces synthesis of select neurotransmitters (dopamine, serotonin) and their respective membrane receptors (Ramboz *et al.* 1998, Centonze *et al.* 2003) known to facilitate lordosis (Apostolakis *et al.* 2004, 2005, Blaustein 2004). Proceptive behaviors also are associated with hormonal modulation of the serial macrocircuits via ER (Pfaff & Schwartz-Giblin 1998), inducible PR (Monks *et al.* 2001), and at least SRC-1 (Monks *et al.* 2003). The present study provides evidence that PR isoform expression plays a role in proceptive behaviors. Proceptive acceptance behaviors and darting coupled with positive lordosis were predominant in PR-A-expressing females under the steroid regimen that promotes lordosis. In contrast, PR-B-expressing females treated with EB+Prog displayed acceptance behaviors (initiating contact with the male and pausing with male contact) but not positive lordosis. Evidence of genotype-specific behavior was also observed for challenge behaviors. Under EB+Prog treatment, PR-BKO females were the only mice to exhibit biting whilst only PRLacZKO females exhibited fighting. Unlike acceptance behaviors, the sum of challenge behaviors for PR-AKO and PR-BKO proceptive behaviors exceeded that for WT mice. For mild avoidance behaviors, vehicle-treated PR-BKO females displayed excessive jumping, an effect that was attenuated by EB alone and in combination with Prog. Regardless of treatment regimen, no other genotype displayed this behavior. Together, the data implicate PR isoforms in the type of proceptive behaviors exhibited and steroid treatments in the frequency of behaviors. Clearly, more studies are needed to further understand these results.

To summarize, the effects of PR isoforms in the VMN appear complex and likely involve target genes for both PR-A and PR-B. The present study implicates PR-A in female reproductive behavior. Colocalization of both PR-A and PR-B RNA was detected in individual neurons

of the vVMN that control the spinal motor neurons circuitry of lordosis-producing muscles of the female. However, there was predominant PR-A expression in these vVMN neurons, whereas PR-B was predominant in adjacent ER-containing neurons that undergo spine remodeling with EB priming. Genotype influenced the type of proceptive behaviors whilst steroid milieu was associated with display frequencies. Finally, GnRH driven self-priming of LH secretion was present in PR-A-expressing but not PR-B-expressing females. Altogether, the putative ability of PR-A to modulate receptivity suggests that it is an integral upstream participant in neuroendocrine function and synchronization of reproductive behavior with ovulation.

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