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Anxiolytic activity of progesterone in progesterone receptor knockout mice

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Abstract

Progesterone is an anxiolytic steroid that could play a role in the regulation of anxiety in women. However, the mechanism by which progesterone decreases anxiety is incompletely understood. Progesterone affects the function of the brain by two distinct mechanisms. Progesterone regulates reproductive behavior by activating intracellular progesterone receptors (PRs). In addition, progesterone is believed to influence neuronal activity through its conversion to allopregnanolone, a neurosteroid that acts as a positive allosteric modulator of $GABA_A$ receptors. The extent to which the anxiolytic action of progesterone requires PRs is uncertain. In this study, we utilized PR knockout (PRKO) mice bearing a targeted null mutation of the PR gene that abrogates the function of both PR-A and PR-B subtypes to determine the requirement for PRs in the anxiolytic actions of progesterone. The absence of PR receptor protein expression in PRKO brain was confirmed by immunocytochemistry. In PRKO mice and their isogenic wild-type (WT) littermates, progesterone administration was associated with a dose-dependent rise in plasma allopregnanolone concentrations and corresponding anxiolytic effects in the elevated plus maze test. PRKO mice exhibited a greater anxiolytic response than WT animals although the allopregnanolone levels were similar in the two genotypes. Allopregnanolone also exhibited anxiolytic effects, but the magnitude of the response was similar in both genotypes. Pretreatment of PRKO mice with finasteride, a 5α-reductase inhibitor that blocks the conversion of progesterone to allopregnanolone, completely prevented the anxiolytic activity of progesterone, but had no effect on the response to allopregnanolone, demonstrating that allopregnanolone (or other 5α-reduced metabolites of progesterone) accounts for the response to the parent steroid hormone. These results provide direct evidence that the anxiolytic action of progesterone does not require PRs. However, PR activation by progesterone may influence the anxiolytic response since PRKO mice were more sensitive to progesterone.

Keywords: Progesterone; Progesterone receptor; Allopregnanolone; Finasteride; $GABA_A$ receptor; Neurosteroid; Anxiety; Elevated plus maze

1. Introduction

Progesterone has long been known to have anxiolytic activity in animals (Fernández-Guasti and Picazo, 1992; Freeman et al., 1993; Reddy and Kulkarni, 1996; Reddy and Kulkarni, 1997a,b,c; Friess et al., 1997; Frye et al., 2000, 2004) and man (Little et al., 1974; Kroboth and McAuley, 1997), although the underlying mechanisms have not been fully elucidated. Progesterone regulates diverse neuroendocrine functions by binding to progesterone receptors (PRs), intracellular receptor proteins that are members of the nuclear receptor superfamily of

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transcription factors (Etgen, 1984; Tsai and O'Malley, 1994; Mulac-Jericevic et al., 2000). Many of the behavioral effects of progesterone are believed to be mediated by PRs that are expressed in hypothalamic and extrahypothalamic cell populations within the brain. In particular, PRs in the hypothalamus and limbic areas are required for the induction of progesterone-dependent reproductive behaviors in females and are also involved in the regulation of maternal and aggressive behaviors (Parsons et al., 1982; Mani et al., 1997; Conneely et al., 2001; Levine et al., 2001). In addition, progesterone can influence behavior through its metabolite allopregnanolone, a neurosteroid that does not interact with PRs but which can rapidly influence brain excitability as a result of its activity as a potent positive allosteric modulator of $GABA_A$ receptors (Rupprecht, 2003; Lambert et al., 2003). Studies in various animal models of anxiety have demonstrated that allopregnanolone has anxiolytic activity (Bitran et al., 1991; Wieland et al., 1991, 1995; Carboni et al., 1996; Brot et al., 1997; Reddy and Kulkarni, 1996; Reddy and Kulkarni, 1997a,b, 1999; Rodgers and Johnson, 1998; Frye et al., 2000; Finn et al., 2003). Moreover, several lines of evidence indicate that the anxiolytic effects of progesterone occur as a result of its conversion to allopregnanolone. Thus, progesterone-induced anxiolytic responses have been found to be correlated with blood and brain allopregnanolone levels and with the degree of facilitation of $GABA_A$ receptor-mediated chloride current responses (Bitran et al., 1993, 1995). In addition, anxiety measures are closely correlated with natural fluctuations in progesterone levels during the ovarian cycle and the corresponding changes in plasma and brain allopregnanolone (Schmidt et al., 1994; Wang et al., 1996; Rapkin et al., 1997; Rubinow et al., 1998; Genazzani et al., 1998; Frye and Bayon, 1998; Concas et al., 1998). Finally, treatment with a 5α-reductase inhibitor that prevents the conversion of progesterone to allopregnanolone eliminates the anxiolytic activity of progesterone (Bitran et al., 1995). However, the anxiolytic activity of progesterone is absent in some but not all anxiety models in mice deficient in type I 5α-reductase (Frye et al., 2004). Notably, there is no reduction in the anxiolytic effects of progesterone in the elevated plus maze, despite the fact that brain allopregnanolone levels are reduced by 60%.

Even if allopregnanolone is primarily responsible for the anxiolytic action of progesterone, PRs are activated when endogenous levels of the hormone rise or when progesterone is administered exogenously. The parent steroid could therefore play a role in the overall anxiolytic response by influencing gene expression relevant to the anxiety behaviors. In addition, although allopregnanolone does not bind directly to PRs (Gee et al., 1988; Rupprecht et al., 1993, 1996), allopregnanolone is capable of indirectly influencing PRs as a result of its in situ conversion to 5α-pregnane steroids that do activate PRs (Rupprecht et al., 1993). Therefore, in the present study, we examined the possible contribution of PRs to the anxiolytic activity of progesterone using progesterone receptor knockout (PRKO) mice. PRs exist in two protein isoforms (PR-A and PR-B) that are transcribed from a single gene. In PRKO mice, both isoforms are functionally ablated by targeting the PR gene in the germ line (Lydon et al., 1995). PRKO mice showed no overt behavioral abnormalities compared with wild-type (WT) animals and the anxiolytic activity of progesterone was not diminished in the knockout animals, providing strong confirmation that PRs are not required for the anxiolytic actions of progesterone.

2. Materials and methods

2.1. Animals

Adult female PRKO and WT mice (25–30 g) were used in the study. The development of PRKO mice has been described previously (Lydon et al., 1995). A breeding colony of PRKO mice was established at the North Carolina State University (NCSU) College of Veterinary Medicine. WT and PRKO mice on a C57BL6/129SvEv hybrid background were housed separately, four to a cage with food and water available ad libitum. Because female PRKO mice are infertile, heterozygous and homozygous PRKO pups were derived by crossing heterozygous females and homozygous males. All behavioral experiments were performed between 9 AM and 5 PM at room temperature. WT mice were used in experiments without regard to the stage in the estrus cycle, while PRKO mice do not cycle. All animal procedures were approved by the NCSU Animal Care and Use Committee.

2.2. Genotyping

Each mouse was identified for the PR genotype using a validated protocol as described previously (Brisken et al., 1998). Briefly, genomic tail-clip DNA was extracted by the phenol/chloroform method and 1 μl of DNA was subjected to PCR amplification using *taq* DNA polymerase (Invitrogen, Carlsbad, CA). PCR was performed by denaturing the DNA at 94 °C for 5 min, followed by 38 cycles of amplification: 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. The following PR-specific primers were used: P1 (5′-TAG ACA GTG TCT TAG ACT CGT TGT TG-3′), P3 (5′-GAT GGG CAC ATG GAT GAA ATC-3′), and a *neo* gene-specific primer, N2 (5′-GCA TGC TCC AGA CTG CCT TGG GAA A-3′). The PCR product was separated by 2% agarose gel electrophoresis and visualized with ethidium bromide

staining using a DNA ladder of 100 bp as marker (MBI Fermentas). A 600 bp DNA band indicated WT (*P1*/*P3* primer mix), a 480 bp band indicated PRKO (*P1*/*N2* primer mix), and both bands for heterozygous animals.

2.3. Immunohistochemistry

An affinity purified anti-PR polyclonal antibody (DakoCytomation) was used for immunohistochemical identification of PR expression in the brain (Mani et al., 1996). Mice were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and postfixed overnight in 4% paraformaldehyde, equilibrated for 48 h in 30% sucrose in PBS at room temperature, and frozen in −70 °C isopentane pre-cooled with dry ice. Serial cryostat sections (30 μm) were cut coronally through the whole cerebral cortex. PR immunoreactivity was identified in frozen sections using the anti-PR antibody. In brief, sections were incubated in PBS containing Triton X-100 and normal serum for 1 h and then in the PBS containing the antibody for 3 days at 4 °C. The immunoreaction product was then detected according to the avidin–biotin complex method of Hsu et al. (1981) using the Vectastin ABC Elite kit (Vector Laboratories, Burlingame, CA). After thorough washing, sections were mounted on gelatin-coated slides, dehydrated in ethanol, and cleared in xylene. The specificity of the primary antibody was checked by omission of the anti-PR antibody or omission of the secondary antibody.

2.4. Elevated plus maze test

The anxiolytic effects of progesterone were evaluated in the elevated plus maze test, a sensitive behavioral assay of anxiety in rodents (Lister, 1987; Kulkarni and Reddy, 1996). The plus maze test exploits the natural conflict that rodents exhibit between exploration of a novel area and aversion to open areas and height. The plus maze was constructed of two open arms (16 × 5 cm) and two enclosed arms (16 × 10 cm) elevated to a height of 25 cm from the floor. Thirty minutes after administration of vehicle (20% β-cyclodextrin) or progesterone (50 and 75 mg/kg, s.c.), each mouse was placed in the center of the plus maze facing an open arm. During the 5-min test period, the number of entries onto the open arms and the enclosed arms were determined. When a mouse entered an open arm, the time was measured. An arm entry was recorded when all four paws entered into an arm. Mice were examined in a quiet room under normal fluorescent room light. The maze was cleaned thoroughly at the end of each test. The plus maze test is based on the observation that rodents exhibit an apparent natural aversion to open and high spaces (Kulkarni and Reddy, 1996). Animals spend more time in the enclosed arms than the open arms and anxiolytic drugs typically increase the proportion of open arm entries in relation to total entries and the time spent on the open arm. In the present study, we calculated the percent of open arm entries with respect to the total number of arm entries in the 5 min observation period. We also measured the time spent on open arms and determined a percentage of the 5 min period that the mouse spent on the open arms. An increased percent of open arm entries and percent of time on open arms is interpreted as an anxiolytic effect. Total arm entries provided an estimate of overall locomotor activity.

2.5. Estimation of allopregnanolone

Mice were anesthetized with an injection of ketamine (100 mg/kg)–xylazine (20 mg/kg) solution and ~1 ml carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000 × *g* for 10 min and stored at −20 °C in 10 ml glass tubes containing 7.5% EDTA solution (68 μl). The concentration of allopregnanolone was quantified by liquid chromatography–mass spectrometry using a Hewlett–Packard (Palo Alto, CA) liquid chromatograph (analytical column: Genesis C18, 4 μm, 3 × 30 mm, Jones Chromatography, Lakewood, CO) and Micromass Quattro II mass spectrometer (Reddy and Rogawski, 2002). Briefly, a 200 μl plasma sample was added to a tube containing evaporated internal standard (5β,3α-pregnane-21-ol-20-one). The steroid and internal standard were extracted with 4 ml hexane. Each sample was analyzed using the atmospheric pressure chemical ionization technique under acidic conditions. A standard curve was plotted using pure allopregnanolone in methanol mixed with 0.2 ml of blank plasma. The detection limit of the assay was 5 ng/ml, with an inter-assay variation of <2%.

2.6. Drugs

Stock solutions of progesterone, finasteride and other steroids for injection were made in 20% hydroxypropyl-β-cyclodextrin (β-cyclodextrin) in water, and additional dilutions were made using normal saline. β-Cyclodextrin vehicle was administered to control animals. Drug solutions were administered s.c. or i.p. in a volume equaling 1% of the animal's body weight. Finasteride was from Steraloids (Newport, RI). All other drugs were obtained from Sigma–Aldrich (St. Louis, MO).

2.7. Data analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Group comparisons of the percent of

time spent in the open arms and the percent of open arm entries in the plus maze test were made using two-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. The significance of differences in the mean plasma concentrations was assessed by one-way ANOVA followed by Dunnett's *t*-test. In the construction of dose-response curves, at least 6 animals were tested at each dose. In all tests, the criterion for statistical significance was $p < 0.05$.

3. Results

3.1. PR expression in brain

PR immunoreactive neurons were observed in the hypothalamus (Fig. 1), hippocampus and various other brain regions of WT mice. The number of immunoreactive neurons was highest in the subregions of the hypothalamus, in accordance with prior studies demonstrating a high density of PR receptors in the mouse hypothalamus (Moffatt et al., 1998). Labeled neurons were present but more sparse in forebrain regions relevant to anxiety, including neocortex, hippocampus and amygdala (Parsons et al., 1982; Wagner et al., 2001) (Table 1). PR immunoreactivity was undetectable in the brains of PRKO mice, consistent with the reported complete absence of PR expression in these animals (Mani et al., 1996).

Table 1
Immunohistochemical determination of PR expression in WT and PRKO mouse brain

Brain region	WT	PRKO
Parietal cortex	+	–
Cingulate cortex	++	–
Hippocampus (CA1 subfield)	+	–
Hippocampus (CA3 subfield)	++	–
Hippocampus (dentate gyrus)	++	–
Basomedial amygdala	+	–
Basolateral amygdala	++	–
Ventromedial hypothalamus	+++	–
Mediobasal hypothalamus	+++	–
Arcuate hypothalamus	+++	–
Thalamus	++	–

PR immunoreactivity was estimated by visual inspection using optical microscopy in 30 μm coronal brain sections from two WT and PRKO mice. The sections were incubated with anti-PR antibody and the reaction product was visualized by avidin–biotin-peroxidase staining. The regions were selected according to Franklin and Paxinos (1997). "–" = absent; "+" = low expression; "++" = moderate expression; "+++" = high expression.

3.2. Anxiolytic activity of progesterone in WT and PRKO mice

The anxiolytic effects of progesterone were examined in WT and PRKO mice in the elevated plus maze. In vehicle-treated WT and PRKO mice, there was no significant difference in percent of open arm entries (WT: 22.67 ± 2.79, PRKO: 19.01 ± 2.29; $p > 0.5$) and percent of time spent on open arms (WT: 8.45 ± 1.78,

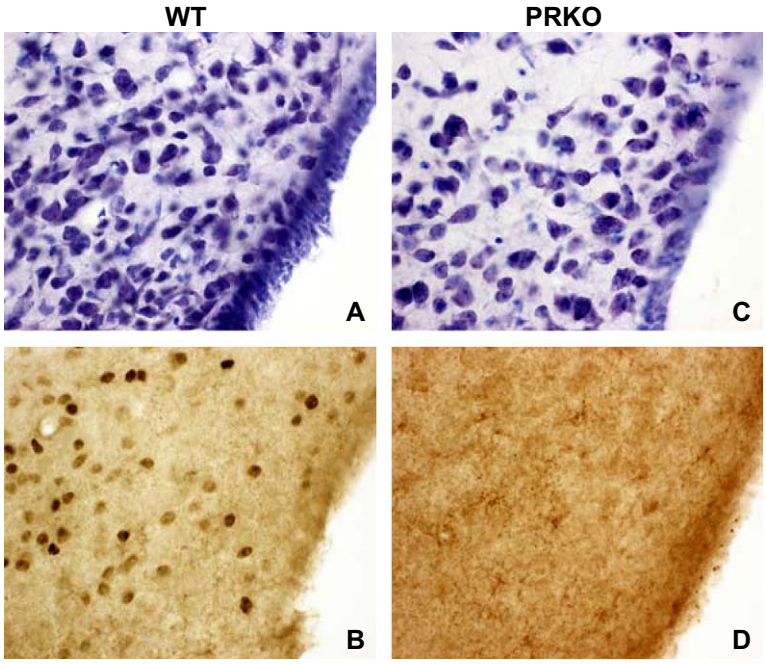

Fig. 1. Photomicrographs of Nissl stained and PR immunoreactive neurons in the medial basal hypothalamus of wild-type (WT) and progesterone receptor knockout (PRKO) mice. Coronal sections (30 μm) were Nissl stained (A and C) or incubated with anti-PR antibody followed by avidin–biotin-peroxidase (B and D). PR immunoreactivity was not detected in sections from PRKO brain (C and D). Images were captured in digital form under identical optical conditions and are displayed at 40× magnification.

PRKO: 4.81 ± 1.52; $p > 0.1$) (Fig. 2A and B), indicating that baseline anxiety levels are similar in the two genotypes. Progesterone treatment (50 and 75 mg/kg, i.p.) was associated with dose-dependent increases in open arm entries and open arm time, indicating an anxiolytic action. The maximal effect of progesterone occurred with the 75 mg/kg dose in both genotypes; higher doses (100 mg/kg) were not associated with greater open arm entries or time on open arms (unpublished). Progesterone at 10 mg/kg or lower doses did not affect performance in the elevated plus maze test. Total number of arm entries did not differ significantly between the vehicle and progesterone-treated WT (vehicle, 6.2 ± 1.3 entries; 50 mg/kg progesterone, 7.7 ± 1.6 entries; 75 mg/kg, 9.8 ± 2.4 entries, $p > 0.1$, Dunnett's t-test) and PRKO mice (vehicle, 6.0 ± 1.4 entries; 50 mg/kg progesterone, 6.4 ± 1.3 entries; 75 mg/kg, 9.5 ± 2.0 entries, $p > 0.1$, Dunnett's t-test), demonstrating that overall locomotor activity was similar in both genotypes.

Fig. 3 compares WT and PRKO mice in terms of the extent to which progesterone increases open arm entries and open arm time. At both the 50 and 75 mg/kg doses, PRKO mice exhibit a greater magnitude anxiolytic response than WT animals (Fig. 3A and B). However, the increase only achieved statistical significance for the 75 mg/kg progesterone dose (both open arm entries and open arm time, $p < 0.05$).

3.3. Allopregnanolone plasma levels in WT and PRKO mice: correlation with anxiolytic activity

To assess the relationship between plasma allopregnanolone and anxiolytic effects, plasma concentrations of allopregnanolone were determined in WT and PRKO mice 30 min after progesterone injections. Progesterone treatment and blood collection were performed on the same animals as used for anxiety testing, but on separate days. As illustrated in Fig. 2C, plasma allopregnanolone concentrations increased in a dose-dependent fashion with increasing progesterone dose (50 and 75 mg/kg, s.c.). There were no significant differences in allopregnanolone plasma concentrations in WT and PRKO mice at either of the two progesterone doses. A significant correlation ($p < 0.01$) was found between the progesterone-induced increase in plasma allopregnanolone levels and the percent of open arm entries in WT ($r = 0.991$) and PRKO mice ($r = 0.995$). There was a similar significant ($p < 0.01$) correlation between the plasma allopregnanolone levels and percent of time spent on open arms in PRKO mice ($r = 0.955$) and their WT littermates ($r = 0.908$). These results indicate a clear dose-dependent relationship between the progesterone-induced rise in plasma allopregnanolone levels and the anxiolytic effects of progesterone.

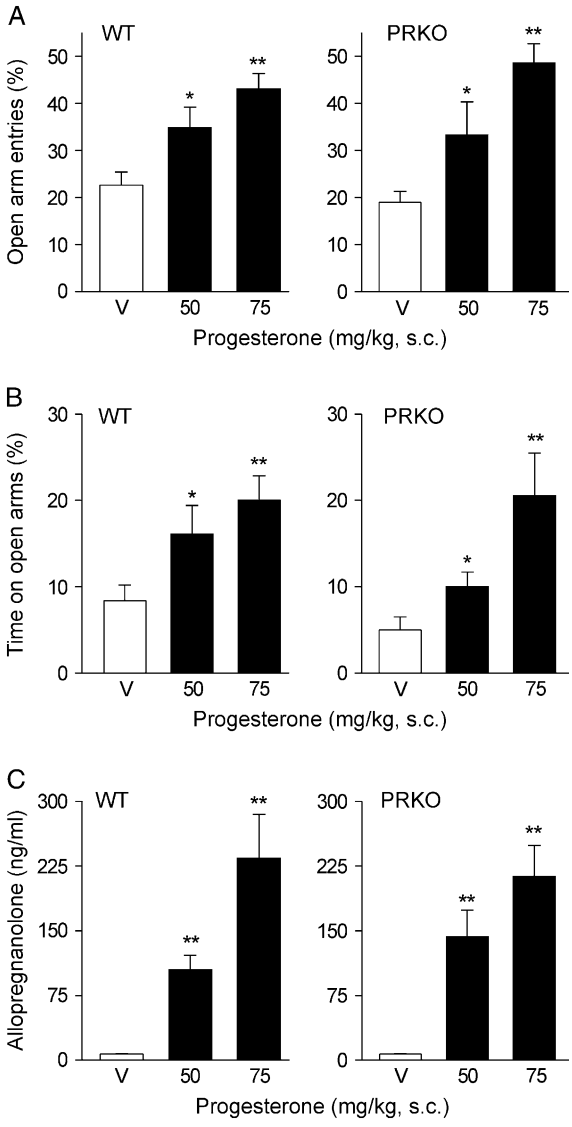

Fig. 2. Anxiolytic effect of progesterone in female WT and PRKO mice in the elevated plus maze test. β-Cyclodextrin vehicle (V) or progesterone (50 and 75 mg/kg, s.c.) was administered 30 min before the plus maze test. In WT mice, progesterone was associated with a significant dose-dependent increase in percent open arm entries [$F(2,19) = 4.78$, $p < 0.02$] (A) and time spent on the open arms [$F(2,19) = 11.21$, $p < 0.01$] (B). PRKO mice also exhibited dose-dependent increases in percent open arm entries [$F(2,19) = 7.41$, $p < 0.01$] (A) and time spent on the open arms [$F(2,19) = 7.71$, $p < 0.01$] (B). Each bar represents mean ± S.E.M. of values from 6 to 9 mice. *$p < 0.05$; **$p < 0.01$ vs. vehicle control (Dunnett's t-test). (C) Plasma allopregnanolone concentrations following vehicle or progesterone administration. Plasma samples were collected 30 min after subcutaneous progesterone injections and allopregnanolone was analyzed by liquid chromatography and mass spectrometry. Allopregnanolone in vehicle-treated mice are below the limit of detection of the assay. There is a significant dose-dependent increase in plasma allopregnanolone [$F(5,33) = 8.21$, $p < 0.001$]. Each bar represents mean ± S.E.M. of samples from 4 to 7 mice. **$p < 0.01$ vs. vehicle control (Dunnett's t-test).

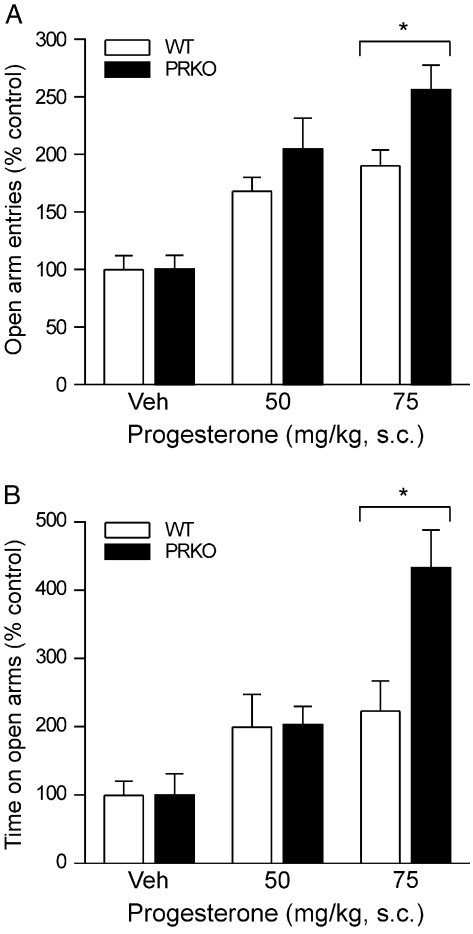

Fig. 3. Comparison of the relative increase in open arm entries (A) and time on open arms (B) for WT and PRKO mice derived from data of Fig. 2. PRKO mice demonstrated enhanced responsiveness to progesterone, but the effect was only significant at the 75 mg/kg dose ($p < 0.05$ for both open time entries and time on open arms). Bars represent the mean ± S.E.M. *percent of control*, calculated for each animal with respect to that animal's control value.

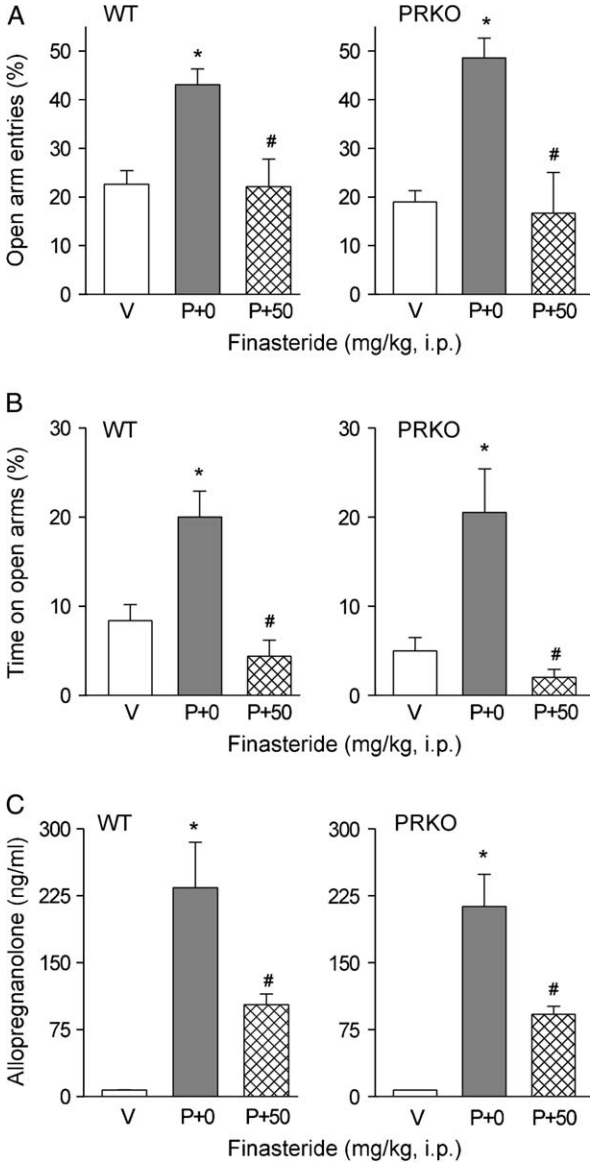

Fig. 4. Finasteride inhibition of the anxiolytic effect of progesterone in female WT and PRKO mice. Progesterone (75 mg/kg, s.c.) significantly increased open arm entries (A) and time spent on the open arms (B) in WT and PRKO animals ("P+0") compared with vehicle-treated animals (V). In animals pretreated with finasteride (50 mg/kg, i.p. 60 min prior to progesterone; "P+50"), open arm entries (A) and time spent on the open arms (B) were not significantly different from the values in vehicle-treated animals. (C) Corresponding plasma allopregnanolone levels in WT and PRKO mice. Mice were subjected to plus maze test 30 min after progesterone injections. Each bar represents the mean ± S.E.M. of data from 6 mice. *$p < 0.01$ vs. vehicle group; #$p < 0.01$ vs. progesterone only group (Dunnett's *t*-test).

3.4. Finasteride reverses the anxiolytic activity of progesterone in WT and PRKO mice: correlation with allopregnanolone levels

To determine whether the anxiolytic effect of progesterone is due to its conversion to the neurosteroid allopregnanolone, PRKO mice were pretreated with finasteride, a 5α-reductase inhibitor that blocks the conversion of progesterone to allopregnanolone (Azzolina et al., 1997). Finasteride (50 mg/kg, i.p.) completely prevented the progesterone-induced increase in percent of open arm entries and time spent on the open arms (Fig. 4A and B). Finasteride alone did not exhibit any intrinsic behavioral activity in the plus maze in PRKO mice (percent open arm entries: 21.0 ± 4.6 control vs. 16.0 ± 3.8 finasteride treatment; percent time spent on open arms: 7.0 ± 2.6 control vs. 6.0 ± 1.3 finasteride treatment, $p > 0.5$; 6 mice/group) as compared to vehicle-treated control mice. As shown in Fig. 4C, finasteride (50 mg/kg) pretreatment significantly (~65%) reduced but did not eliminate progesterone-induced increases in plasma allopregnanolone concentrations in WT and PRKO mice.

3.5. Anxiolytic activity of allopregnanolone in WT and PRKO mice

The observation that finasteride pretreatment inhibits the anxiolytic activity of progesterone indicates that the antianxiety action of progesterone results from conversion to allopregnanolone. To develop further support for this conclusion, allopregnanolone itself was examined in the elevated plus maze. At doses of 2 and 5 mg/kg (s.c.), allopregnanolone caused a significant, dose-dependent increase in open arm entries (Fig. 5A) and open arm time (Fig. 5B) compared to vehicle-treated control mice. Analysis of total arm entries, an index of locomotor activity, indicated no significant effect of allopregnanolone treatment in WT and PRKO mice (data not shown). Moreover, pretreatment with finasteride (50 mg/kg, i.p.) did not affect allopregnanolone (5 mg/kg, s.c.)-induced changes in percent open arm entries in WT (51.0 ± 7.5 allopregnanolone alone vs. 45.0 ± 5.5 allopregnanolone and finasteride treatment; $p > 0.1$; 6–8 mice/group) or PRKO mice (48.0 ± 7.6 allopregnanolone alone vs. 42.0 ± 6.3 allopregnanolone and finasteride treatment; $p > 0.1$; 6–8 mice/group). Similar results were found for the analysis of percent time on open arms in WT (30.0 ± 6.9 allopregnanolone alone vs. 44.0 ± 9.3 allopregnanolone and finasteride treatment; $p > 0.1$; 6–8 mice/group) or PRKO mice (26.0 ± 7.2 allopregnanolone alone vs. 37.0 ± 7.1 allopregnanolone and finasteride treatment; $p > 0.1$; 6–8 mice/group). These results demonstrate that finasteride has no effect on the anxiolytic activity of allopregnanolone in the plus maze test in WT and PRKO mice.

As shown in Fig. 6, there were no significant difference in the effects of allopregnanolone on open arm entries or time on open arms between WT and PRKO animals.

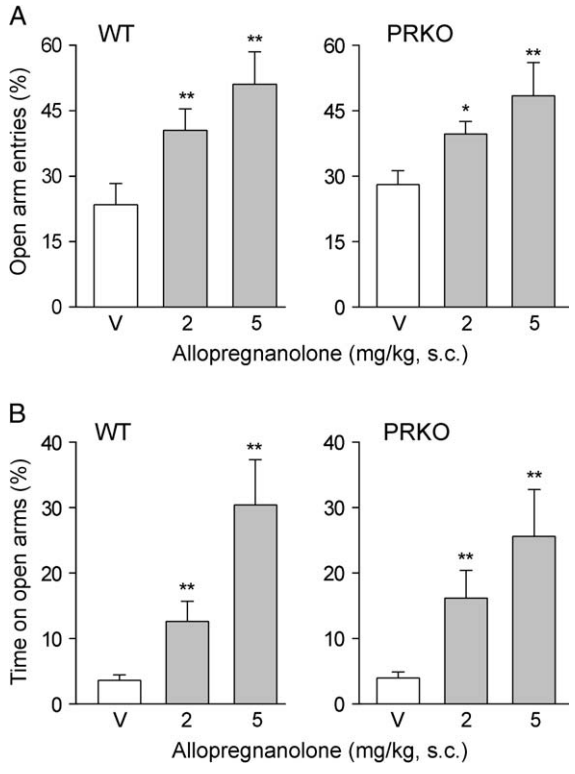

Fig. 5. Anxiolytic effect of allopregnanolone in WT and PRKO mice in the elevated plus maze test. β-Cyclodextrin vehicle (V) or allopregnanolone (2 and 5 mg/kg, s.c.) was administered 15 min before the plus maze test. In WT mice, allopregnanolone was associated with a significant dose-dependent increase in percent open arm entries [$F(2,16) = 5.26$, $p < 0.01$] (A) and time spent on the open arms [$F(2,16) = 4.21$, $p < 0.05$] (B). PRKO mice exhibited comparable dose-dependent increases in percent open arm entries [$F(2,16) = 9.68$, $p < 0.01$] (A) and time spent on the open arms $F(2,16) = 5.38$, $p < 0.01$] (B). Each bar represents mean ± S.E.M. of values from 6 to 8 mice. *$p < 0.05$; **$p < 0.01$ vs. vehicle control (Dunnett's t-test).

4. Discussion

The observation that the activity of progesterone in the elevated plus maze is undiminished in PRKO mice

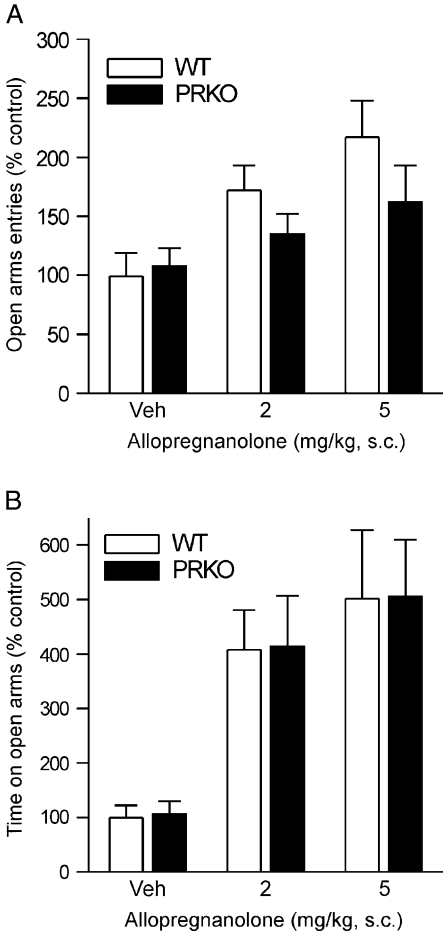

Fig. 6. Comparison of the relative increase in open arm entries (A) and time on open arms (B) for WT and PRKO mice derived from data of Fig. 5. WT and PRKO mice demonstrated similar responsiveness to allopregnanolone ($p > 0.05$ for both open time entries and time on open arms). Bars represent the mean ± S.E.M. *percent of control*, calculated for each animal with respect to that animal's control value.

compared to WT littermates demonstrates that PRs are not required for the anxiolytic action of progesterone. This is the same conclusion as that reached by Bitran et al. (1995) using a pharmacological antagonist of PRs. However, not only was the anxiolytic effect of progesterone preserved in the PRKO mice, but it was actually larger than in WT controls (the difference was significant only at the 75 mg/kg dose of progesterone). The greater activity of progesterone in the anxiety model used here corresponds with our recent report that progesterone exhibits a substantial increase in anticonvulsant potency in PRKO mice (Reddy et al., 2004). In the present study and in the prior one, there was no difference in the potency of allopregnanolone in PRKO mice and controls, indicating that the variation in progesterone sensitivity does not reside in altered sensitivity to the metabolite. Also, in the present study and in our prior one, plasma allopregnanolone determinations demonstrated that the altered sensitivity in the PRKO animals cannot be explained by differences in the metabolism of progesterone to allopregnanolone since the plasma levels achieved in the two genotypes were comparable. Additionally, there was no difference in the behavior of untreated PRKO mice in the elevated plus maze compared with untreated WT animals and, indeed, there is little or no difference in the baseline plasma concentrations of progesterone between WT and PRKO mice (Chappell et al., 1997). Thus, the enhanced progesterone responsiveness is not likely to be due to underlying differences in anxiety levels or to effects resulting from chronic differences in endogenous progesterone. Rather, we have proposed that exogenous progesterone acting via PRs may cause reduced sensitivity to progesterone-derived allopregnanolone in animals that express PRs, but that this mechanism is inoperative in animals lacking PR, thus accounting for their enhanced sensitivity (Reddy et al., 2004). Moreover, it is conceivable that progesterone could be anxiogenic through actions at PRs, so that ablation of PRs would permit a greater anxiolytic effect via allopregnanolone. However, this seems unlikely as "anxiety levels" at baseline or after progesterone alone or progesterone given in combination with finasteride were not significantly greater in WT than in PRKO mice. Thus, our data do not support the concept that progesterone activation of PRs, either acutely or chronically is anxiogenic. It is notable that progesterone at the doses used in the present study did not nonspecifically affect the locomotor activity as measured by the total number of arm entries. This provides support for interpreting the progesterone-induced changes in plus maze behavior as anxiolytic effects.

Bitran et al. (1995) have proposed that the anxiolytic activity of progesterone in the elevated plus maze requires in vivo reduction of the parent steroid hormone to allopregnanolone. Our results are consistent with this hypothesis. We found that progesterone administration was associated with a dose-dependent elevation in plasma allopregnanolone levels and that the magnitude of the plasma concentration increase was well correlated with the response in the plus maze. Finasteride pretreatment completely prevented the effects of progesterone in the plus maze. This was associated with a $\sim$65% decrease in plasma allopregnanolone. Finasteride is a selective 5α-reductase inhibitor (Azzolina et al., 1997) that blocks the metabolism of progesterone to the intermediate 5α-dihydroprogesterone, thus reducing its availability for subsequent conversion to allopregnanolone by ubiquitous 3α-hydroxysteroid oxidoreductases (3α-HSOR). The initial 5α-reduction represents the rate-limiting step in allopregnanolone biosynthesis from progesterone (Russell and Wilson, 1994). In rodents, finasteride inhibits both the type I 5α-reductase isoenzyme that is widely distributed throughout the body and is most abundant in the liver, as well as the type II isoenzyme that is primarily expressed in target tissues for androgens, such as the prostate and seminal vesicles (see Rogawski and Reddy, 2004, for references). Although brain exhibits substantially lower 5α-reductase activity than peripheral tissues, there is evidence that allopregnanolone can be synthesized in situ (Mellon et al., 2001; Dong et al., 2001). The type I isoenzyme is the predominant form in rat brain, although the type II form is transiently expressed during late fetal and early postnatal life (Poletti et al., 1998) and can be induced in the female brain by progesterone (Matsui et al., 2002). In our experiments, the activity of progesterone in the plus maze was completely blocked by finasteride, indicating that concentrations of allopregnanolone were reduced below the threshold for anxiolytic activity. However, the plasma level values must be interpreted cautiously as they may not reflect brain levels (or more specifically the concentrations available to brain $GABA_A$ receptors) (Corpechot et al., 1997). Nevertheless, allopregnanolone is a highly lipophilic steroid that can readily cross the blood-brain barrier and there is evidence that brain allopregnanolone levels parallel plasma levels (Bitran et al., 1993; Smith et al., 1998a,b; Frye and Bayon, 1998). Finasteride alone did not affect plus maze behavior, indicating that it does not have nonspecific actions apart from inhibition of 5α-reductase activity. In addition, this observation suggests that basal levels of 5α-reduced steroids including allopregnanolone do not modulate anxiety levels under ordinary circumstances. The anxiolytic activity of allopregnanolone was unaffected by finasteride, confirming that finasteride is not acting by an action unrelated to inhibition of 5α-reductase. Overall, the results strongly support the conclusion that conversion to allopregnanolone is required for the anxiolytic effects. We and others have previously come to similar conclusions regarding other

central nervous system actions of progesterone, including the anticonvulsant and sedative activity (Kokate et al., 1999; Frye et al., 2002; Reddy et al., 2004; Rhodes et al., 2004).

Recently, Frye et al. (2004) reported that the plus maze activity of progesterone was undiminished in homozygous Srd5a1 mice bearing a targeted null mutation in the type I 5α-reductase gene. (Progesterone did have reduced activity in other tests of anxiety in the knockout animals.) This observation contrasts with our present results and those of Bitran et al. (1995) in which pharmacological inhibition of 5α-reductase eliminated the activity of progesterone in the plus maze test. The differing results could be explained if the pharmacological 5α-reductase inhibitors produced a greater reduction in brain allopregnanolone. In fact, Frye et al. reported that brain allopregnanolone levels were only reduced 60% in the type I 5α-reductase deficient mice. Type II 5α-reductase presumably accounts for the allopregnanolone in the knockout animals. Moreover, it is conceivable that induction of brain type II 5α-reductase by the exogenously administered progesterone (Matsui et al., 2002) could balance the defect in the type I enzyme.

In addition to allopregnanolone, several other progesterone metabolites could potentially contribute to the anxiolytic activity of progesterone. For example, 3α-hydroxy-pregn-4-ene-20-one, a neurosteroid with moderate $GABA_A$ receptor modulatory activity, can be formed by the direct action of 3α-HSOR on progesterone when 5α-reductase is inhibited by finasteride (Morrow et al., 1990; Reddy and Kulkarni, 2000). However, this steroid is unlikely to be of substantial importance to the action of progesterone in the present study since finasteride completely prevented its anxiolytic activity. Pregnanolone, the 5β-isomer of allopregnanolone, also has anxiolytic activity (Wieland et al., 1995; Bitran et al., 1999; Gomez et al., 2002). There is uncertainty as to whether pregnanolone is an endogenous metabolite inasmuch as 5β-reductase activity has not been demonstrated in peripheral tissues or brain (Kondo et al., 1994). In any case, given the fact that finasteride fully blocks the anxiolytic activity of progesterone, pregnanolone could only contribute in a minimal way to the anxiolytic activity of progesterone. These various considerations indicate that progesterone's actions on anxiety behavior largely depend on allopregnanolone and not other potential metabolites.

Allopregnanolone is believed to exert its various behavioral actions and also its protection against seizures through positive modulation of $GABA_A$ receptors (Lambert et al., 2003; Rogawski and Reddy, 2004). This conclusion is supported by comparison of the plasma allopregnanolone levels associated with anxiolytic effects as determined in the present study with the concentrations known to influence $GABA_A$ receptors. Concentrations of allopregnanolone as low as 1 nM are active at $GABA_A$ receptors (Cooper et al., 1999; Belelli et al., 2002) and a concentration of 30 nM doubles the GABA-evoked current in cultured hippocampal neurons (Kokate et al., 1994). If brain allopregnanolone levels are similar to the mean plasma levels (30 min after a 50 mg/kg anxiolytic dose of progesterone allopregnanolone levels were ~100 ng/ml = 31 nM), the receptor concentrations would be in a range that would produce substantial augmentation of $GABA_A$ receptor responses. This concentration of allopregnanolone is higher but still comparable to physiological serum concentrations, which in women range from 2 to 4 nM, depending upon the phase of the menstrual cycle (Wang et al., 1996; Rapkin et al., 1997; Genazzani et al., 1998; Monteleone et al., 2000). As noted, plasma concentrations might not fully reflect brain levels because of local synthesis. Nevertheless, it is reasonable to conclude that the levels of allopregnanolone formed from anxiolytic doses of progesterone are sufficient to influence $GABA_A$ receptor function and this likely accounts for the effects observed in our behavioral model.

In summary, progesterone has anxiolytic-like effects in the elevated plus maze model in PRKO mice, providing strong evidence that PRs are not required for its anxiolytic activity. The 5α-reductase inhibitor finasteride completely blocks the anxiolytic activity of progesterone in PRKO mice by decreasing allopregnanolone synthesis. Thus, the anxiolytic activity of progesterone is mediated through its metabolic conversion to allopregnanolone, which is a potent $GABA_A$ receptor-modulating neurosteroid. Endogenous allopregnanolone formed from progesterone might be relevant to the regulation of anxiety in women and could play a role in pathological anxiety, such as in the premenstrual syndrome.

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