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Research report

Anesthetic effects of progesterone are undiminished in progesterone receptor knockout mice

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Abstract

Progesterone has sedative and anesthetic effects but the underlying molecular mechanisms remain unclear. The two possible mechanisms by which progesterone affects the function of the brain include binding to intracellular progesterone receptors (PR) and metabolism to $GABA_A$ receptor-modulating neurosteroids. In this study, PR knockout (PRKO) mice were used as model to study the role of PRs in the anesthetic activity of progesterone. The progesterone-induced anesthetic activity was undiminished in female PRKO mice (ED_{50}, 172 mg/kg) as compared to their wild-type littermates (ED_{50}, 167 mg/kg). The progesterone-induced anesthetic activity was highly correlated with increased plasma allopregnanolone levels. Pretreatment of PRKO mice with the 5α-reductase inhibitor finasteride significantly reduced the progesterone-induced anesthetic activity. Allopregnanolone also evoked dose-dependent anesthetic activity in PRKO mice, which was similar to those of wild-type mice. Thus, the anesthetic activity of progesterone is not mediated by its interaction with PRs. The neurosteroid allopregnanolone partially mediates the anesthetic activity of progesterone by potentiation of $GABA_A$ receptor function.

Theme: Neurotransmitters, Modulators, Transporters, and Receptors
Topic: GABA receptor

Keywords: Progesterone; Neurosteroid; Allopregnanolone; Progesterone receptor; Sleep; Righting reflex

1. Introduction

Progesterone has long been known to elicit sedative, anxiolytic, and anesthetic activity [2,3,6,16,21,28]. However, the molecular mechanisms underlying the central nervous system effects of progesterone remain unclear. Progesterone affects the function of the brain by at least two distinct mechanisms: binding to intracellular steroid receptors and metabolism to 5α-reduced neuroactive steroids. The physiological effects of progesterone are mediated by intracellular progesterone receptors (PRs), which are nuclear transcription factors that mediate the action of progesterone in target cells [13,19]. Apart from the hypothalamus, PRs are expressed in many brain regions in females including the hippocampus, neocortex, and limbic areas [4,12,14,20]. The PR is composed of two protein isoforms, PR-A and PR-B subtypes that are expressed from the same PR gene. In addition, progesterone is a substrate of the 5α-reductase enzyme, which converts progesterone into the neurosteroids 5α-dihydroprogesterone and allopregnanolone in the brain. There is emerging evidence to support the hypothesis that the anesthetic effects of progesterone are caused by its conversion to allopregnanolone, which is an allosteric positive modulator of $GABA_A$ receptors [8,11,17,18,29]. Consistent with its allosteric activity at $GABA_A$ receptors, allopregnanolone induces sleep in a benzodiazepine-like fashion [9,10]. Although allopregnanolone binds poorly to PRs, it may indirectly affect PRs by intracellular oxidation to 5α-dihydroprogesterone, a moderately potent PR agonist [26,27]. Thus, the role of PRs in mediating the sedative–anesthetic activities of progesterone

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and allopregnanolone has not been addressed directly by gene targeting approaches.

In the present study, we evaluated the role of PRs in the anesthetic actions of progesterone in PR knockout (PRKO) mice. The PRKO mouse model was developed by a targeted null mutation of the PR gene that abrogates the function of both PR-A and PR-B subtypes [13]. In addition, the 5α-reductase inhibitor finasteride was used to block the critical biochemical pathway of progesterone's conversion to allopregnanolone [1]. Our results confirm that the PR is not involved in the anesthetic activity of progesterone, which is presumably mediated by the $GABA_A$ receptor-modulating neurosteroid allopregnanolone.

2. Materials and methods

2.1. Animals

Adult female PRKO ($PR^{-/-}$) and wild-type (WT) ($PR^{+/+}$) mice (25–30 g) were used in the study. The development of PRKO mice has been described previously [13]. A breeding colony of PRKO mice was established at the North Carolina State University (NCSU) College of Veterinary Medicine. WT and PRKO mice, with a C57BL6/129SvEv hybrid background, were housed separately four to a cage with food and water available ad libitum. 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 [25]. Briefly, genomic DNA from mouse tails was extracted by the phenol/chloroform method and 1 μl of DNA was subjected to PCR amplification using *taq* DNA polymerase (Invitrogen, Inc). PCR was performed by denaturing the DNA at 94 °C for 5 min, followed by 37 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 presence of primer amplified PCR product was detected by agarose gels and visualized by ethidium bromide fluorescence. We observed the presence of a 600 bp DNA band for WT ($PR^{+/+}$), a 480 bp band for homozygous ($PR^{-/-}$), or both bands for mice heterozygous for PR gene ($PR^{+/-}$).

2.3. Loss of the righting reflex

Anesthetic effect of progesterone was evaluated using the loss of the righting reflex (LORR) method [8]. Mice were given an intraperitoneal injection of progesterone (75–300 mg/kg) and placed on their backs. The time interval between loss and return of the righting reflex was then recorded. Animals were judged to have regained the reflex when they could right themselves three times within a 30-s interval. Each dose of progesterone was tested in groups of 6 to 8 mice. Each mouse (WT or PRKO) was used only once in the LORR experiment. Thus, each group of animals received a single dose of either progesterone or allopregnanolone. Control group received vehicle (20% β-cyclodextrin solution). In the construction of dose–response curves, at least 6 mice were used at each dose point. After recording the onset and duration of LORR response, animals were promptly euthanized.

2.4. Estimation of allopregnanolone

Mice were anesthetized with an injection of a 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–tandem mass spectrometry (LC–MS–MS) using a Hewlett-Packard (Palo Alto, CA) liquid chromatograph (analytical column: Genesis C18, 4 μm, 3 × 30 mm, Jones Chromatography, Lakewood, CO) and a Micromass Quattro II mass spectrometer [23]. 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.

2.5. Drugs

Stock solutions of progesterone, allopregnanolone, and finasteride 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 group. Drug solutions were administered sc or ip in a volume equaling 1% of the animal's body weight. Finasteride was procured from Steraloids (Newport, RI). All other drugs were obtained from Sigma (St. Louis, MO).

2.6. Data analysis

The effect of progesterone and allopregnanolone on the latency or duration of LORR response was evaluated in a dose-dependent fashion. The percent of LORR occurrence at each dose point was calculated from group of 6–8 mice. ED_{50} values (the doses at which 50% of tested animals lost LORR) with 95% confidence limits in the anesthetic test were determined by log-probit analysis using the Litchfield and

Wilcoxon procedure. In the construction of dose–response curves, at least 6 animals were tested at each dose. The significance of differences between steroid dose–response curves in the percent LORR occurrence was assessed with the Litchfield and Wilcoxon χ^2 test. Group comparisons in the percentage of animals exhibiting LORR response were made with the Fisher's exact probability test. To determine if PRKO mice had different latency or duration measures than WT mice, mean latency of LORR, and duration of LORR responses were analyzed by two-way ANOVA with genotype × dose or treatment as two variables. Mean allopregnanolone concentrations were assessed by one-way ANOVA followed by Dunnett's t test. In all tests, the criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Anesthetic activity of progesterone in PRKO mice

The anesthetic activity of progesterone was assessed by the LORR behavioral assay in female WT and PRKO mice. As shown in Fig. 1A, progesterone (75–300 mg/kg, ip) administration induced a dose-dependent LORR occurrence in WT mice, indicating an anesthetic effect of progesterone. The progesterone-induced anesthetic activity was undiminished in PRKO mice as compared to WT littermates (Fig. 1A). The ED_{50} values derived from these curves are listed in Table 1. The occurrence and duration of LORR to progesterone (200 mg/kg) was similar in WT and PRKO mice (Figs. 2A and B). Progesterone administration resulted in dose-dependent elevations of plasma allopregnanolone concentrations in WT and PRKO mice (Fig. 1B). Plasma allopregnanolone concentrations were comparable in both genotypes. The plasma concentrations of allopregnanolone significantly correlated ($r = 0.92$, $P < 0.01$) with the percent LORR occurrence both in PRKO and WT mice.

3.2. Effect of finasteride on the anesthetic activity of progesterone in PRKO mice

If progesterone induces anesthesia indirectly through the action of its neuroactive metabolites, then inhibition of progesterone metabolism by the 5α-reductase inhibitor finasteride would be expected to affect its anesthetic activity. Consistent with this hypothesis, finasteride pretreatment (75 mg/kg, ip), 60 min before progesterone injection (200 mg/kg, ip), significantly prevented the occurrence of progesterone-induced LORR in WT and PRKO mice (Fig. 2A). Finasteride significantly reduced the duration of progesterone-induced LORR responses in both PRKO mice and WT controls (Fig. 2B). Finasteride (75 mg/kg, ip) alone did not produce any noticeable behavioral effects in control WT and PRKO mice. Moreover, the finasteride dose used in the present study (75 mg/kg) has previously been shown to significantly (~60%) decrease plasma allopregnanolone levels [24].

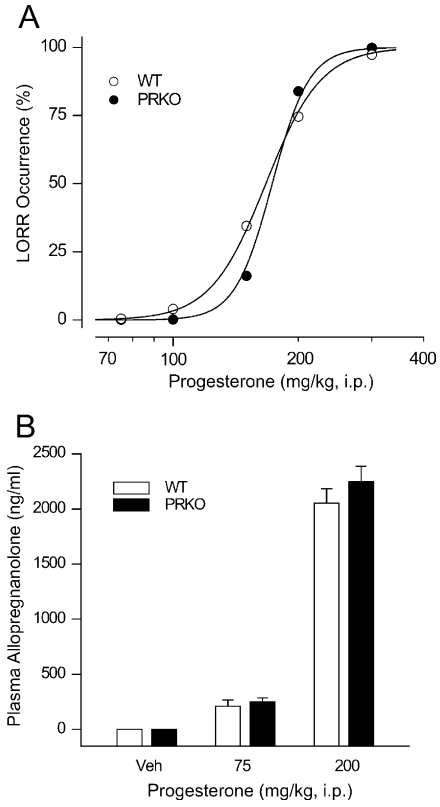

Fig. 1. Progesterone-induced anesthetic activity in PRKO mice. (A) Dose–response relationship of progesterone (75–300 mg/kg, ip)-induced loss of the righting reflex (LORR) in female WT and PRKO mice. The occurrence of LORR was determined as described in Section 2.3. Each point represents the data from six to eight animals. (B) Dose-dependent elevation of plasma allopregnanolone concentrations in WT and PRKO mice. Plasma samples were collected 30 min after progesterone (75–200 mg/kg, ip) administration in separate groups of mice. Data represent mean ± SEM (n = 4 to 7 mice per group).

3.3. Anesthetic activity of allopregnanolone in PRKO mice

If allopregnanolone is a major neuroactive metabolite that mediates progesterone action on anesthesia, then direct administration of allopregnanolone would be expected to produce effects similar to those of progesterone. In agreement with this notion, allopregnanolone administration (10–50 mg/kg, sc) elicited dose-dependent LORR responses in PRKO mice similar to that of WT animals (Fig. 3A). The allopregnanolone (30 and 50 mg/kg)-induced latency and duration of LORR in PRKO and WT mice was shown in Figs. 3B and C. Two-way ANOVA indicated that there was no significant difference in either latency of the LORR or duration of the LORR response with allopregnanolone (30 and 50 mg/kg) in the PRKO mice than in WT mice. However, a modest enhanced duration was evident in PRKO mice as compared to WT mice. The time course for LORR responses indicates that progesterone exhibited a slow onset of LORR (44 ± 7 min in WT; 65 ± 12 min in PRKO mice) and a sustained duration of LORR (121 ± 22 min in WT; 110 ± 16 min in PRKO mice). In contrast, the allopregnanolone-induced latency of LORR was shorter

Table 1
Anesthetic ED_{50} values (mg/kg) of progesterone and allopregnanolone in female WT and PRKO mice

Genotype	Progesterone	Allopregnanolone
WT (+/+)	167 (142–197)	24 (18–29)
PRKO (−/−)	172 (149–200)	29 (22–37)

Numbers in parentheses are 95% confidence limits. The ED_{50} values were obtained from log-probit fits to the data presented in Figs. 1A and 3A.

(~15 min) and it diminished during the 40-min period after injection in both genotypes (Fig. 3). These results are compatible with the possibility that progesterone serves as precursor for the synthesis of allopregnanolone or other 5α-reduced neurosteroids.

4. Discussion

The key observation of this study is that the anesthetic activity of progesterone is undiminished in PRKO mice, providing direct evidence that the PR does not mediate the anesthetic activity of progesterone. Recent studies have shown that progesterone induces sleep in humans and animals that is highly comparable to that caused by clinically used benzodiazepines [6,9]. In the present study, the role of PRs in the anesthetic activity of progesterone was investigated using the LORR as the behavioral model. Because the relationship between the LORR model to human sleep is not well defined, it cannot be concluded that PRs do not participate in the regulation of sleep in physiological conditions such as during the menstrual cycle and pregnancy.

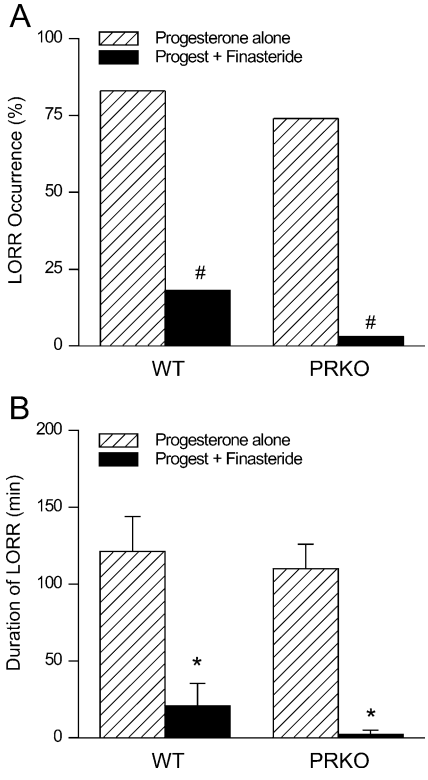

Fig. 2. Effect of finasteride on progesterone-induced anesthetic activity. (A) The incidence of progesterone (200 mg/kg, ip)-induced LORR was similar in female WT and PRKO mice, but was significantly reduced by pretreatment with finasteride (75 mg/kg, ip). (B) The duration of progesterone (200 mg/kg, ip)-induced LORR was similar in WT and PRKO mice, but was significantly decreased by pretreatment with finasteride (75 mg/kg, ip). Finasteride was administered 60 min before progesterone. Data represent mean ± SEM (n = 6 to 8 mice per group). $^{\#}P < 0.05$ vs. progesterone alone (Fisher's exact probability test); $^{*}P < 0.05$ vs. progesterone alone (Dunnett's t test).

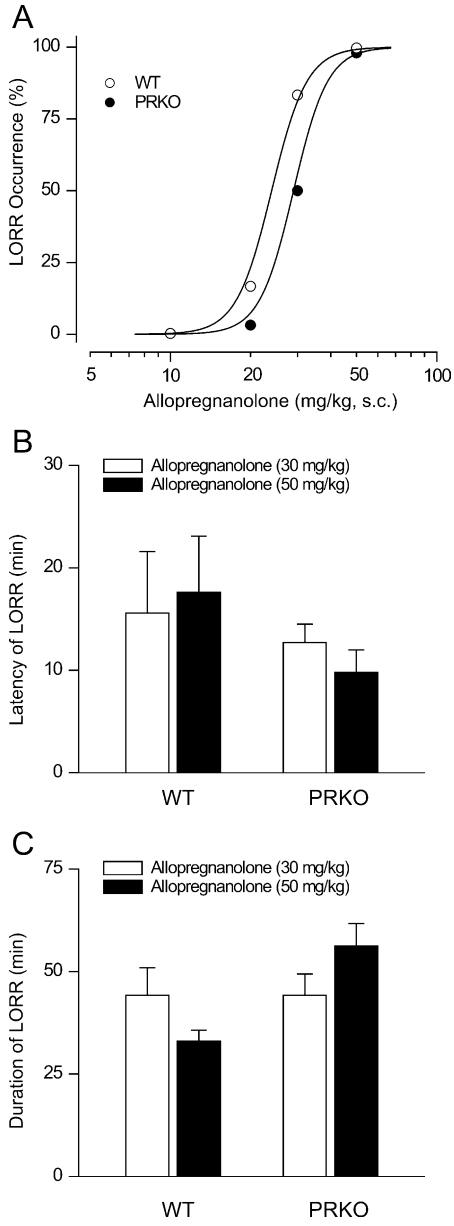

Fig. 3. Allopregnanolone-induced anesthetic activity in PRKO mice. (A) Dose–response relationship of allopregnanolone (10–50 mg/kg, sc)-induced percent LORR occurrence in female WT and PRKO mice. Each point represents the data from six to eight animals. (B) Allopregnanolone (30 and 50 mg/kg, sc)-induced latency of LORR in WT and PRKO mice. (C) Allopregnanolone (30 and 50 mg/kg, sc)-induced duration of LORR in WT and PRKO mice. (B and C) Data represent mean ± SEM (n = 6 to 8 mice per group).

The present study shows that the progesterone-derived neurosteroid allopregnanolone at least partly mediates the anesthetic activity of progesterone. The following observations support this contention. First, progesterone has powerful anesthetic activity in PRKO mice, which are deficient in both PR-A and PR-B subtypes. Second, progesterone administration resulted in a dose-dependent elevation of allopregnanolone levels in the plasma in PRKO mice and WT controls. Third, the anesthetic activity of progesterone was significantly inhibited by finasteride pretreatment in WT and PRKO mice. Finasteride is a selective 5α-reductase inhibitor that blocks the metabolism of progesterone to dihydroprogesterone, an intermediate required for allopregnanolone synthesis [1]. Finally, direct administration of allopregnanolone induced similar anesthetic activity in WT and PRKO mice. Therefore, our results support the conclusion that the anesthetic activity of progesterone results not from effects on the PR but rather depends on the conversion of progesterone to 5α-reduced metabolites, principally allopregnanolone. These results are consistent with previous studies that demonstrated a key role for the progesterone-derived allopregnanolone in the anesthetic activity of progesterone [8,16,17].

The metabolism of progesterone to neuroactive steroids occurs both in peripheral tissues and in the brain [15]. It is hypothesized that the active metabolite responsible for the anesthetic activity of progesterone is mainly allopregnanolone. Allopregnanolone is a neurosteroid because it is synthesized de novo in the brain [5,15,22]. Allopregnanolone is synthesized from progesterone by two sequential enzymatic A-ring reductions. The 5α-reductase first converts progesterone to 5α-dihydroprogesterone, which is then reduced further by 3α-hydroxysteroid oxidoreductase to allopregnanolone. The 5α-reduction is an irreversible and rate-limiting reaction, while 3α-reduction is reversible and occurs more readily. In the present study, the possible involvement of neuroactive steroids such as 5β-pregnanolone, 3α-hydroxy-pregn-4-ene-20-one, and 20α-dihydroprogesterone in the anesthetic activity of progesterone is not determined directly. However, these steroids could be synthesized from progesterone by alternative metabolic pathways when the main 5α-reductase pathway is inhibited by finasteride [22]. This is consistent with our observations that finasteride did not achieve complete blockade of progesterone anesthesia. Thus, it is possible that these neuroactive steroids could be partially responsible for the anesthesia induced by progesterone.

The neurosteroid allopregnanolone has been demonstrated to induce sleep in animals and humans comparable to those of agonistic $GABA_A$ receptor modulators [6,9,10]. The $GABA_A$ receptor is an important target for allopregnanolone, but it may also regulate gene expression via the PR after intracellular oxidation to dihydroprogesterone, which is a moderately potent PR agonist [25,26]. In the present study, allopregnanolone has similar anesthetic activity in WT and PRKO mice, suggesting that PRs are not involved in the anesthetic activity of allopregnanolone. The anesthetic effects of allopregnanolone could be due to its interaction with $GABA_A$ receptors. This contention is mainly supported by the observation that allopregnanolone has specific binding sites on the postsynaptic $GABA_A$ receptor chloride channel that are distinct from the binding sites for GABA, benzodiazepines, and barbiturates [7]. Like benzodiazepines, allopregnanolone is an extremely potent positive allosteric modulator of $GABA_A$ receptors [11]. Low nanomolar concentrations of allopregnanolone are sufficient to activate the $GABA_A$ receptor channel by allosteric potentiation of GABA. At high concentrations (>10 μM), allopregnanolone can directly activate $GABA_A$ receptor channels in the absence of GABA [11].

The measurements of plasma allopregnanolone levels allow us to directly correlate the progesterone-induced elevation in allopregnanolone concentrations with its anesthetic activity in PRKO mice. There is a significant correlation between the plasma allopregnanolone concentrations achieved with various progesterone doses and corresponding anesthetic activity. The mean plasma concentration of allopregnanolone determined after progesterone (200 mg/kg) administration in WT (2050 ng/ml = 630 nM) and PRKO mice (2200 ng/ml = 680 nM) is within the concentration range sufficient to potentiate $GABA_A$ receptor function [10]. Brain concentrations of allopregnanolone highly correlate with those of plasma levels [5], but actual brain levels could be slightly higher because of local biosynthesis. Although these concentrations are supraphysiological, there are indications that physiological concentrations of allopregnanolone may influence sleep–wake behavior [9,10]. Overall, these findings support the hypothesis that the hypnotic effects of progesterone are mediated via its metabolism to neuroactive steroids that subsequently augment $GABA_A$ receptor function.

In conclusion, the present study shows that progesterone has undiminished anesthetic activity in PRKO mice, providing strong evidence that PRs are not involved in the anesthetic effects of progesterone. Progesterone dose dependently elevates the concentrations of allopregnanolone. Pretreatment with finasteride reduces the progesterone-induced anesthetic activity. These results confirm that neurosteroids such as allopregnanolone mediate the anesthetic effects of progesterone by potentiation of $GABA_A$ receptor function.

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