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Disease-modifying activity of progesterone in the hippocampus kindling model of epileptogenesis

Doodipala Samba Reddy[*], **Omkaram Gangisetty**, and **Seema Briyal**
Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M Health Science Center, College Station, TX 77843, USA

Abstract

Progesterone (P) is an endogenous anticonvulsant hormone. P is being evaluated as a treatment for epilepsy, traumatic brain injury, and other complex neurological conditions. Preclinical and clinical studies suggest that P appears to interrupt epileptogenic events. However, the potential disease-modifying effect of P in epileptogenic models is not widely investigated. In this study, we examined the effects of P on the development of hippocampus kindling in female mice. In addition, we determined the role of progesterone receptors (PR) in the P's effect on the kindling epileptogenesis utilizing PR knockout (PRKO) mice. P, at 25 mg/kg, did not affect seizures and did not exert sedative/motor effects in fully-kindled mice. P treatment (25 mg/kg, twice daily for 2 weeks) significantly suppressed the rate of development of behavioral kindled seizure activity evoked by daily hippocampus stimulation in wild-type (WT) mice, indicating a disease-modifying effect of P on limbic epileptogenesis. There was a significant increase in the rate of 'rebound or withdrawal' kindling during drug-free stimulation sessions following abrupt discontinuation of P treatment. A washout period after termination of P treatment prevented such acceleration in kindling. PRKO mice were kindled significantly slower than WT mice, indicating a modulatory role of PRs in seizure susceptibility. P's effects on early kindling progression was partially decreased in PRKO mice, but the overall (~2-fold) delay in the rate of kindling for the induction of stage 5 seizures was unchanged in PRKO mice. Moreover, the acute anticonvulsant effect of P was undiminished in fully-kindled PRKO mice. These studies suggest that P exerts disease-modifying effects in the hippocampus kindling model at doses that do not significantly affect seizure expression and motor performance, and the kindling-retarding effects of P may occur partly through a complex PR-dependent and PR-independent mechanism.

Keywords

Progesterone; epileptogenesis; kindling; disease-modifying; neurosteroid; progesterone receptor; seizure

[*]***Correspondence should be addressed to:*** D. Samba Reddy, Ph.D., R.Ph., Associate Professor, Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M Health Science Center, 228 Reynolds Medical Building, College Station, Texas 77843-1114, USA, Tel: 979-862-2852; Fax: 979-845-0790, reddy@medicine.tamhsc.edu.

1. Introduction

Epilepsy is a chronic condition characterized by recurrent unprovoked seizures. Epilepsy affects about 3 million people in the United States, including 1 million women that are of childbearing age (Engel et al., 2007). Antiepileptic drugs (AEDs) are the mainstay for the treatment of epilepsy. Despite the availability of over two dozen AEDs, about one-third of people with epilepsy show seizures that are not controlled to even the best available treatment. Many people with epilepsy need long-term drug therapy that is often associated with debilitating side effects and drug interactions. Presently, there is no specific drug for curing epilepsy (Jacobs et al., 2009). The mechanisms underlying development of epilepsy are not very well understood. The term 'epileptogenesis' is used to describe the complex plastic changes in the brain that, following a precipitating insult or injury, convert a normal brain into a brain debilitated by recurrent seizures (Pitkänen et al., 2009). There is a desperate need for drugs that truly prevent the development of epilepsy ('antiepileptogenic agents') or alter its natural course to delay the appearance or severity of epileptic seizures ('disease-modifying agents'). A number of clinical trials show a lack of antiepileptogenic efficacy of AEDs, including phenytoin and carbamazepine, in patients at high risk for developing epilepsy (Temkin, 2001).

The kindling model of epileptogenesis has provided a conceptual framework for developing prophylactic treatment to prevent the onset of epilepsy or stop its progression (Goddard et al., 1969; McNamara et al., 1992). Kindling is the most widely used model to screen for drugs with disease-modifying efficacy (Löscher, 2002), although it is difficult to find a human correlate of this process because kindling does not lead to epilepsy. However, the lack of antiepileptogenic efficacy of phenytoin and carbamazepine in patients is highly consistent with studies in the kindling model (Wada et al., 1978; Albertson et al., 1984; Silver et al., 1991). Previous studies have shown that only a few drugs, including valproate, exert true antiepileptogenic or disease-modifying effects in the kindling model in that kindling acquisition is significantly retarded after termination of drug treatment (Silver et al., 1991; McNamara et al., 1992). Several newer AEDs such as topiramate, lamotrigine, and lacosamide have been evaluated for disease-modifying activity, but these AEDs had limited or weak efficacy (Walker et al., 2002; Nissinen et al., 2004). Despite levetiracetam's robust efficacy on the kindling epileptogenesis (Löscher et al., 1998), it did not produce either antiepileptogenic or disease-modifying effects in a post-status epilepticus (SE) model of epilepsy (Brandt et al., 2007). These studies lead to the conclusion that the conventional AEDs do not exert a true antiepileptogenic effect. The mechanisms involved in epileptogenesis after injury involve an interaction of acute and delayed anatomic, molecular, and physiological events that are both complex and multifaceted (Walker et al., 2002). Hence, pharmacotherapies should be targeted at the multiple factors that contribute to the epileptogenic cascade, to maximize the likelihood of a successful prevention.

Progesterone (P) is an appealing hormone for prophylactic interventions on epilepsy development, due to its multifunctional modulatory actions in the brain. P is an endogenous anticonvulsant hormone. P has long been known to have antiseizure activity in animal models (Selye 1942; Craig, 1966; Kokate et al., 1999; Frye and Scalise, 2000; Reddy et al., 2004), and in clinical studies (Bäckström et al., 1984; Herzog, 1995; 1999). Therefore, women with epilepsy are prone to seizures in response to decreased levels of P during perimenstrual periods (Herzog et al., 1997; Reddy, 2009). Presently P is being evaluated in an NIH-sponsored, multicenter clinical trial as a treatment for epilepsy in women (Herzog et al., 2008). Indeed, the incidence of epilepsy is generally lower in women than in men (Hauser et al., 1993; Christensen et al., 2005; McHugh and Delanty, 2008). This gender difference could be caused by ovarian hormones such as P. Although P is known to inhibit stimulation-evoked seizures in kindling models (Holmes and Weber, 1984; Mohammad et al., 1998; Lonsdale et al.,

2006), P has not been investigated widely for its potential disease-modifying effect in epileptogenic models. In the amygdala kindling model, P has been shown to impair epileptogenesis in male rats (Holmes and Weber, 1984; Edwards et al., 2001).

Previous studies have shown that P supports the normal development of neurons, and that it reduces the extent of brain damage after traumatic brain injury (TBI) (Roof et al., 1994; Cutler et al., 2005; 2007). It has been observed in animal models that females have reduced susceptibility to TBI and this protective effect has been hypothesized to be caused by increased circulating levels of P in females (Roof and Hall, 2000; Meffre et al., 2007). A number of additional studies have confirmed that P has neuroprotective effects (Gibson et al., 2008). Promising results have also been reported in human clinical trials. Recently, two clinical studies have evaluated P as a treatment for moderate to severe TBI (Wright et al., 2007; Xiao et al., 2008). P is highly efficacious in reducing disability and death in TBI. P has neuroprotective properties in acute models of ischemic injury, stroke, and astroglial dysfunction (Koenig et al., 1995; Jiang et al., 1996; He et al., 2004).

P targets multiple molecular and cellular mechanisms relevant to epileptogenesis, and therefore, P may be a natural disease-modifying agent. P's cellular actions are mediated by the progesterone receptors (PRs), which are expressed in the hypothalamus, neocortex, hippocampus and limbic areas (Brinton et al., 2008). P is an intermediate precursor for the synthesis of neurosteroids (Reddy, 2009). P's antiseizure activity is mediated mainly by its conversion to allopregnanolone, a neurosteroid and positive modulator of GABA-A receptors with broad-spectrum antiseizure properties (Belelli et al., 1989; Kokate et al., 1999; Frye et al., 2002; Kaminski et al., 2004; Reddy et al., 2004). Neurosteroids have unique binding sites on GABA-A receptors (Gee et al., 1995; Hosie et al., 2006). They modulate both synaptic and extrasynaptic GABA-A receptors and their modulatory action is highly sensitive at δ-subunit-containing extrasynaptic GABA-A receptors (Herd et al., 2007). Moreover, neurosteroids can modulate PRs via intracellular metabolism to analogs that bind to PRs (Rupprecht et al., 1993). P may modulate signaling cascades of inflammation, apoptosis, neurogenesis and synaptic plasticity (Patel, 2004; Vezzani, 2005; Stein and Sayeed, 2010), and therefore, P may exert disease-modifying effects on epileptogenesis.

In this study, we investigated the disease-modifying effects of P on hippocampus kindling epileptogenesis in female mice. In addition, we sought to determine the role of the PR in P's effect on kindling development using PR knockout (PRKO) mice. Our results indicate that P has disease-modifying effects that may occur through a complex mechanism.

2. Materials and Methods

2.1. Animals

Adult female mice (3–5 months old) weighing approximately 25–30 g were used in the study. The generation of PR WT (+/+) and PRKO (−/−) mice by a PR null mutation has been described previously (Lydon et al., 1995; Reddy et al., 2004). PRKO mice lack both the PR-A and PR-B isoforms that are transcribed by the PR gene. These mice were maintained in a hybrid C57BL6/129SV background. Animals were housed separately, four to a cage and were fed mouse chow and water *ad libitum*. Genotype was confirmed by PCR using mouse tail genomic DNA (Reddy et al., 2005). Animals were not checked for ovarian cycle stages due to chronic experimental (kindling) protocol that lasted longer than the 5-day cycle. However, changes in seizure susceptibility are not evident among phases of the estrous cycle in kindled animals (Wahnschaffe and Löscher, 1992). All procedures were performed in strict compliance with the *NIH Guide for the Care and Use of Laboratory Animals*, under a protocol approved by the Institutional Animal Care and Use Committee.

2.2. Hippocampus kindling

The kindling epileptogenesis was induced by daily hippocampus stimulations (Rogawski et al., 2001; Reddy et al., 2004). Mice were anesthetized by intraperitoneal injections of ketamine (100 mg/kg) and xylazine (20 mg/kg), and a stimulation-recording bipolar electrode (Plastic-One, Inc.) was stereotaxically implanted in the right ventral hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura) and anchored with dental acrylic to three screws. After a postoperative recovery period of at least 1 week, the electrographic afterdischarge (AD) threshold was determined by application of a 1s train of 1 ms biphasic rectangular pulses at 60 Hz beginning at 25 μA. Additional stimulations increasing by 25 μA were given at 5 min intervals until an electrographic AD lasting at least 5 s was detected using the digital EEG system (Astro-Med, West Warwick, RI). Kindling stimulations (1-ms pulses, 60 Hz frequency, 1s duration) were applied following standard protocol, consisting of a once-daily stimulation at 125% of AD threshold using an isolated pulse stimulator (A-M Systems). Stimulations were delivered daily until stage 5 seizures were elicited on three consecutive days. The AD duration was acquired from the hippocampal electrode using Axoscope 8.0 software (Axon Instruments). Behavioral seizures were rated according to the Racine's scale (1972), as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or head nodding; stage 2, chewing and head nodding (wet dog shakes); stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, falling.

2.3. Drug treatment and kindling studies

The overall experimental protocol for drug treatment and kindling stimulations is illustrated in Fig.1. In the kindling development study (protocol #1), P (25 mg/kg) was injected subcutaneously, 30-min prior to stimulation sessions each morning, on days 1 to 14. Animals received a second dose in the evening. [P has a very short half-life, <0.5 h, therefore, multiple doses are needed to maintain adequate plasma levels]. Stimulations were applied daily during P treatment and during the P-free (withdrawal) period. This treatment design has been utilized for testing drugs against kindling development (Dürmüller et al., 1994;Rogawski et al., 2001). In the second study (protocol #2), animals received P treatment (25 mg/kg, s.c., twice daily) for 14 days, followed by a 1-week washout period. Animals were stimulated during P treatment and again after the washout period. Stock solutions of P (Steraloids, Inc.) for injection were made in 15% β-cyclodextrin solution, and additional dilutions were made using normal saline. Control animals received vehicle injections. β-Cyclodextrin alone, at concentrations as high as 50%, failed to affect kindled seizures. Stimulation was continued on a daily basis, seven days per week during drug-treatment sessions and drug-free sessions. Kindling stimulation was continued until stage 5 seizures were elicited on three consecutive days. In a separate group of WT and PRKO mice, kindling stimulation was delivered daily (five days per week) without drug treatment until stage 5 seizures elicited on three consecutive days. These mice were used for drug testing when they consistently exhibited stage 5 seizures with stimulation, which is considered the "fully kindled" state. To examine the ability of P to suppress the expression of kindled seizures, the kindled mice underwent a 3-day test protocol. On day 1, they were verified to exhibit stage 5 seizures. On day 2, a subcutaneous P injection was administered 30 min prior to stimulation. On day 3, the animals were stimulated again without P pretreatment. During each test session, the behavioral seizure score and the afterdischarge duration were recorded as indices of drug efficacy.

2.4. Sedation/motor impairment test

Evaluation for sedation/motor impairment was carried out using an inverted screen test that determines an animal's ability to support its own body weight by grasping a grid (Coughenour et al., 1977). Thirty minutes after the P injection, mice were placed on a horizontally oriented grid (consisting of parallel 1.5-mm-diameter rods situated 1 cm apart), and the grid was

inverted. Animals that fell from the grid within 10 s were scored as positive for sedation/motor impairment. Vehicle-treated mice never fell from the grid within 10 s.

2.5. Estimation of P and allopregnanolone levels

Mice were anesthetized with isoflurane, and 0.5 ml carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000g for 10 min and stored at −20°C. The concentration of allopregnanolone was quantified by liquid chromatography-mass spectrometry as described previously (Reddy et al., 2004). Briefly, a 200 μl plasma sample was added to a tube containing evaporated internal standard. The steroid and internal standard were extracted with 4 ml of hexane. Each sample was analyzed using the atmospheric pressure chemical ionization technique under acidic conditions. Plasma concentrations of P were analyzed by an immunoassay (Antech GLP). The detection limit of the assay was <0.2 ng/ml.

2.6. Data analysis

Group data was expressed as the mean ± standard error of the mean (SEM); differences in seizure stages between groups were compared with the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U*-test. Comparison of means of the afterdischarge duration between groups was made with one-way analysis of variance, followed by the unpaired two-tailed Student's *t*-test. Comparison of the mean percentage inhibition of seizure stage and afterdischarge duration in fully kindled animals was made by the Wilcoxon signed ranks test and the paired two-tailed Student's *t*-test, respectively. In all statistical tests, the criterion for statistical significance was $p<0.05$.

3. Results

3.1. Effect of P on seizure expression and motor performance

To determine the optimum dose of P for testing against hippocampus kindling development, the activity of P in suppressing against kindled seizures was evaluated in fully-kindled WT mice. As shown in Fig.2, P suppressed the expression of behavioral seizures and reduced the AD duration at high dose (100 mg/kg) but did not significantly affect seizure activity at low doses (25 mg/kg). Impairment in motor performance, assessed as time on an inverted screen bar, occurred in mice receiving 100 mg/kg of P but not at 25 mg/kg (Fig.2C), which was selected for kindling development studies.

3.2. Effect of P on hippocampus kindling development in WT mice

The development of hippocampus-kindled seizures in control mice, and in mice that had received P (25 mg/kg) treatment from day 1 to 14 is shown in Fig.3. There was no significant group difference in initial AD threshold (control: 85 ± 10 μA; P-treatment: 84 ± 20 μA). In control WT mice, there was a progressive increase in behavioral seizure intensity with all animals achieving stage 5 after 13 stimulations (Fig.3A). The electrographic AD duration did not vary significantly during the initial stimulation sessions but continued stimulation was associated with an increase in AD duration in control animals (Fig.3B). In WT animals, P produced a marked retardation of development of kindling epileptogenesis, as evident by significant suppression of behavioral seizures in comparison to controls at the corresponding stimulation sessions (Fig.3A). However, P did not significantly affect the mean AD duration (Fig.3B). Table 1 presents the mean number of stimulations to achieve various seizure stages. The P-treatment group showed marked suppression of the development of partial (stage 2 and 3) and generalized (stage 4 and 5) seizures (Table 1). The rate of kindling, measured by the number of stimulations required to elicit stage 5 seizures, was significantly inhibited in mice treated with P compared to controls (Fig.4), indicating disease-modifying effect of P on the limbic epileptogenesis.

3.3. Comparison of the rate of kindling progression during P treatment and discontinuation

To compare the progression of kindling in the P-treatment period (from day 1 to 14) with that in the subsequent P-free period (from day 14 to stage 5), the rate of kindling in the two phases was determined in WT mice (Fig.1, protocol #1). As shown in Fig.5A, there was no significant difference in the rate of kindling in two phases in control animals. However, there was a significant decrease in the rate of behavioral kindling progression during the P-treatment period (disease-modifying effect) and a significant increase in rate during drug-free stimulation sessions following abrupt discontinuation of P-treatment period (rebound or withdrawal effect) (Fig.5A). These results suggest that P inhibition of kindling development is associated with a substantial rebound or withdrawal in the apparent rate of kindling when the P is discontinued abruptly.

3.4. Comparison of rate of kindling progression during P treatment and washout period

To compare the progression of kindling in the P-treatment period (from day 1 to 14) with that in the subsequent period after a 1-week washout, the rate of kindling in the two phases was determined in WT mice (Fig.1, protocol #2). As shown in Fig.6, there was a significant decrease in the rate of kindling progression during the P treatment period. Following the washout period, the rate of kindling did not differ significantly between the P-treated and control groups. The washout period prevented the acceleration of the kindling rate that was observed in animals following the abrupt withdrawal of P (Fig.5A), indicating that P withdrawal may have counteracted the disease-modifying effect of P treatment.

3.5. Effect of P on hippocampus kindling development in PRKO mice

To determine the role of PRs in P's activity against kindling epileptogenesis, we utilized the PRKO mice that lack both the PR-A and PR-B subtypes in the brain. We first compared the progression of kindling in PRKO mice with WT mice (Table 1, Fig.3). PRKO mice showed slower kindling development as revealed by reduced behavioral seizure stages and shorter AD duration than WT controls at corresponding stimulation sessions (Fig.3CD). The rate of kindling for induction of stage 5 seizures was significantly ($p<0.05$) delayed in PRKO mice compared to WT mice (Fig.4), indicating a modulatory role of PRs in seizure susceptibility.

In PRKO mice, P (25 mg/kg, twice daily for 2 weeks) treatment did not affect the behavioral seizures (Fig.3C) and AD duration (Fig.3D) during early kindling progression, but there was significant retardation of behavioral seizure progression at the later period. The PRKO mice that received P showed marked suppression of the development of generalized (stage 4 and 5) seizures (Table 1). P significantly (~2-fold) inhibited the overall rate of kindling for induction of the fully kindled state (third stage 5 seizures) in PRKO mice (Fig.4), which was comparable to its effect in WT mice. No significant group differences in initial AD threshold were observed in PRKO mice (control: 90 ± 14 μA; P-treatment: 71 ± 12 μA). In contrast to WT mice, there was no evidence of rebound increase in kindling rate in PRKO mice following discontinuation of P treatment (Fig.5B). Thus, these results indicate that the disease-modifying effect of P may occur partly through PR-dependent and PR-independent mechanisms.

3.6. Plasma levels of P during chronic treatment

To determine the pharmacokinetic factors in P's effects on the kindling development, we determined the plasma levels of P in control and 7-day chronically P treated mice. Plasma levels of P were determined 30 min after the administration of P (25 mg/kg, s.c.) in WT and PRKO mice. Plasma P levels were similar in control WT and PRKO mice (Fig.7). There were no significant differences in the mean P levels achieved between WT and PRKO mice during chronic P treatment (Fig.7). These results suggest that any lasting effects of P on kindling after chronic administration in WT mice were not due to its accumulation.

3.7. Effect of P on seizure expression in fully-kindled PRKO mice

In fully-kindled PRKO mice showing stage 5 seizures, P (50 and 100 mg/kg) produced a dose-dependent inhibition of behavioral seizures and electrographic AD durations (Fig.8B). The P-induced inhibition of behavioral seizure activity was greater in PRKO mice as compared to their WT controls (Fig.8A). In PRKO mice, finasteride (100 mg/kg, ip) treatment, given 1 h prior to P (100 mg/kg) injections, significantly reduced plasma allopregnanolone levels (P alone: 854 ± 47 ng/ml; P+finasteride: 386 ± 55 ng/ml), and reversed the P's suppression of behavioral seizure stage (P alone: 0.5 ± 0.22; P+finasteride: 4.6 ± 0.4) and AD duration (P alone: 16 ± 1.4 s; P+finasteride: 26 ± 2.5 s). Thus, these results confirm that the acute anticonvulsant activity of P mainly occur through PR-independent mechanisms, most likely via P's conversion to the neurosteroid allopregnanolone.

4. Discussion

The present study shows that P can retard or suppress kindling epileptogenesis in female mice. The key observations of this study are: (i) Treatment with P, at dosages that do not have anticonvulsant efficacy and that do not shorten the afterdischarge, significantly delays the development of hippocampus kindling epileptogenesis; (ii) Abrupt withdrawal of P accelerates the kindling rate that may counteract the disease-modifying effect of P treatment; (iii) PRKO mice show slower kindling development than WT mice; and (iv) The kindling-retarding effect of P is partially decreased in PRKO mice. Collectively, these studies suggest that P has disease-modifying effects that could delay or limit the severity of limbic epileptogenesis. However, there are certain caveats that must be considered in regards to the interpretation of these findings. First, the results apply only to the kindling model used in this study. Second, this study supports the disease-modifying potential of P to the extent that kindling development represents a model of limbic epileptogenesis. However, the relationship of this model to human epilepsy is not well validated. Third, the relevance of the dosages of P that were used in this study in relation to those required for prophylactic treatment is uncertain. Nevertheless, our findings are consistent with previous reports on the effect of P on epileptogenesis (Edwards et al., 2001) and kindled seizures (Mohammad et al., 1998; Lonsdale and Burnham, 2003).

P exerts disease-modifying effect on kindling

In the present study, we investigated the development of hippocampus kindling during P treatment and P-free period or 1-week after washout following the termination of P treatment (Fig.1). Treatment with P, at a dose that does not affect seizure expression and does not exert sedative/motor performance, significantly inhibited the development of hippocampus kindling epileptogenesis. P's retardation of behavioral manifestations of kindled seizures without affecting electrographic AD duration is consistent with the possibility that P inhibits the generalization of the seizure discharge from the hippocampus focus, and thereby modulates progression of epileptogenesis. This data confirms previous observations of Holmes and Weber (1984) and Edwards et al. (2001) who have reported that P significantly delays amygdala kindling in rats. Although our results are consistent with these and other earlier reports (Edwards et al., 1999), there are major differences on several aspects of the study. We utilized the mouse hippocampus kindling paradigm and tested the low dose P that did not affect seizure expression and AD activity, whereas previous studies have utilized gonadectomized male rats (Edwards et al., 2001) or immature male rats (Holmes and Weber, 1984). Although limbic seizures may affect hormones (Edwards et al., 1999), the kindling attenuating effect of P is not entirely dependent on the endocrine milieu (Holmes et al., 1984). Moreover, plasma P levels are similar between genotypes during a chronic treatment period, demonstrating that pharmacokinetic factors such as drug accumulation cannot account for the P's protective activity in WT mice. These findings may have translational relevance to interrupt epileptogenesis in patients at risk for developing epilepsy, especially in people with stroke,

TBI, brain infection, status epilepticus and related injury-induced triggering factors (Pitkänen et al, 2009;Stein and Sayeed, 2010).

There are general limitations in our studies, especially issues on the relevance of the kindling model. Kindling and post-SE models are the most commonly used for studies of epileptogenesis and for testing antiepileptogenic and neuroprotective agents (Löscher, 2002, Pitkanen, 2002). Kindling is a widely used model to test the efficacy of drugs that suppress the development of epileptogenesis (McNamara et al., 1992). Several studies have suggested that drugs that inhibit the development of kindling may have disease-modifying properties in humans. However, there is no strong evidence on the validity of the kindling model for testing antiepileptogenic agents, because traditional kindling does not lead to epilepsy (only after >50–100 stimulations, ~50% of animals develop spontaneous seizures). The conventional AEDs do not exert a true disease-modifying effect, except valproate and levetiracetam that retard kindling during the treatment period and following drug withdrawal (Silver et al., 1991; Löscher et al., 1998). Despite promising effects in the kindling model, levetiracetam failed to exhibit antiepileptogenic action in post-SE model of epileptogenesis (Brandt et al., 2007). Although post-SE models with a latent period could possibly be more relevant to human epilepsy, they are more labor intensive and are not highly suitable for extensive screening of potential antiepileptogenic agents. Despite concerns on validity and generalization to other models, kindling is a very useful model for rapid identification of disease-modifying drugs.

Abrupt withdrawal of P accelerates kindling

Our results indicate that the stage of kindling achieved during P-treatment and after the termination of P treatment in WT mice is significantly below that of the control level obtained with a corresponding number of stimulations. Thus, there is strong retardation of kindling with P treatment. However, experimental agents that inhibit the development of kindling epileptogenesis may accelerate the rate of kindling development when the treatment was terminated abruptly (Rogawski et al., 2001). Kindling rebound may occur during drug-free period or drug effects on kindling development attenuate over time; this would imply that the irreversible processes of kindling development continue during the drug-treatment period, but that drug treatment prevents the behavioral manifestations. This is highly unlikely in case of P effects on epileptogenesis because of low doses that did not affect behavioral seizure expression. However, our results clearly demonstrate that following termination of treatment with P, there was a marked (~2-fold) increase in the rate of kindling development as monitored by the expression of kindled seizures in WT mice (Fig.5A). This is attributed to abrupt discontinuation of P or P-derived neurosteroids (withdrawal effect) because withdrawal from chronic treatment with P and neurosteroids has been shown to trigger increase in seizure susceptibility (Moran and Smith, 1998; Reddy et al., 2001). Following the washout period, the kindling rate is similar between both groups. Therefore, continuous P treatment or tapered P discontinuation is desirable for persistent inhibition of kindling epileptogenesis.

Potential mechanisms of the disease-modifying activity of P

The molecular mechanisms underlying P's attenuating effect on kindling development remains unknown. There are several potential mechanisms by which P could inhibit epileptogenesis, including activation of PRs, synthesis of neurosteroids, modulation of oxidative cascades, and promoting neuroprotection. The effect of P on the early kindling progression is reduced in mice lacking PRs, which provide evidence that PRs may be partly involved in P's disease-modifying effects. These findings are consistent with the role of PRs in P inhibition of epileptiform activity in the hippocampus (Edwards et al., 2000). The PRKO mice exhibited a marked delay in the rate of kindling, suggesting that PRs are not involved in the later part of the kindling progression. However, the extent to which PRs mediate the P's disease-modifying effect remains unclear because we unexpectedly observed that PRKO mice exhibit delayed kindling,

indicating that PRs may influence seizure susceptibility. Collectively, the disease-modifying effect of P may occur through a complex mechanism partly involving PR-dependent and PR-independent pathways.

P is rapidly metabolized into pregnanediol, allopregnanolone, and other hydroxylated derivatives. P is a major substrate for endogenous neurosteroids such as allopregnanolone, which could mediate P's attenuating effects on kindling epileptogenesis. This possibility is supported by emerging evidence that neurosteroids can retard the development of spontaneous seizures in post-SE models of epileptogenesis (Biagini et al., 2006, 2009). In a rapid kindling model in adult mice, pre-treatment with finasteride (50 mg/kg) significantly blocked P's inhibition of epileptogenesis (Reddy and Mohan, 2010), suggesting that neurosteroids may be involved in P's antiepileptogenic effects. Neurosteroid increase in tonic inhibition in the hippocampus could inhibit the spread of the seizure discharge from the hippocampus focus and thereby suppress the rate of development of behavioral kindled seizure activity without affecting the focal electrographic discharges. Increased tonic inhibition by allopregnanolone is shown to impair the NMDA receptor-mediated excitability in the hippocampus (Shen et al., 2010). It is likely that such a mechanism may underlie the P's disease-modifying effects in the kindling model.

In addition, P's modulation of inflammation is suggested as an appealing mechanism because pro-inflammatory molecules and oxidative signaling has been found to be activated in animal models of epilepsy (Vezzani, 2005; Patel, 2004). P has pleiotropic effects on inflammation and cell growth/survival (He et al., 2004; Stein and Sayeed, 2010), that may contribute to its attenuating effects on epileptogenesis. P inhibits secreted phospholipase A2 enzyme, a very high level target in the inflammatory cascade that has been shown to induce neurodegeneration through glutamate release (Yagami et al., 2002; DeCoster et al., 2002). P has been shown in numerous preclinical models to be a neuroprotective after injury (Roof et al., 1994; Koenig et al., 1995; Jiang et al., 1996; Cutler et al., 2007). Recently, two clinical studies have evaluated P as a treatment for moderate to severe TBI (Wright et al., 2007; Xiao et al., 2008). The P was administered over a period of 3 or 5 days beginning within 8 or 11 hours of the injury. In both studies, the groups receiving P had significantly fewer deaths than those receiving placebo. In addition, there was evidence of improved functional outcomes in the P-treated groups, suggesting that P is highly efficacious in reducing morbidity and mortality in TBI, which is a leading cause of epilepsy in adults and military persons.

In summary, our results demonstrate that P, in addition to exerting anticonvulsant activity, has the potential to retard kindling-induced epileptogenesis. To the extent that hippocampus kindling development represents a model of limbic epileptogenesis, this study supports the potential of P as a prophylactic treatment in people at high risk for developing epilepsy or in early stages of the disease. The disease-modifying effect of P occurs through a complex mechanism partly involving PR-dependent and PR-independent pathways including P's conversion to neurosteroids that have been recently demonstrated to retard epileptogenesis in a spontaneous model (Biagini et al., 2006) and exert neuroprotective effects in a TBI model (Meffre et al., 2007).

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ABBREVIATIONS

P	progesterone
PR	progesterone receptor

PRKO	PR knockout
WT	wild-type
AED	antiepileptic drug
TBI	traumatic brain injury
AD	afterdischarge
SE	status epilepticus

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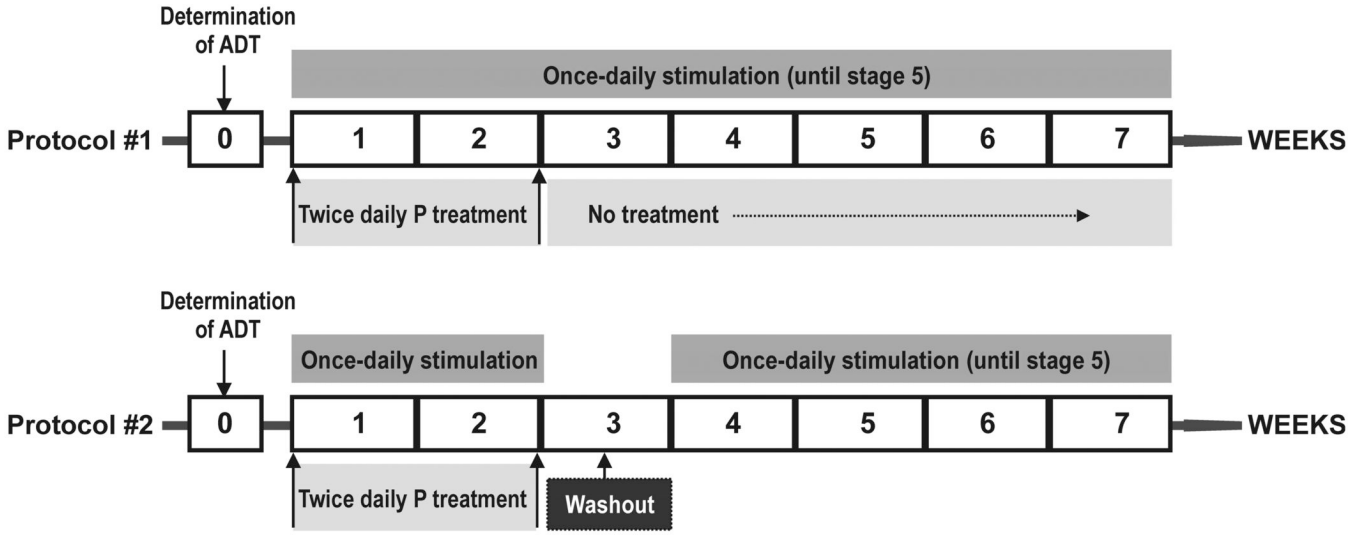

Fig. 1. Study design

In protocol #1, animals were treated with P (25 mg/kg, s.c., twice daily) for 2 weeks, followed by a P-free (withdrawal) period. In protocol #2, animals were treated with P for 2 weeks, followed by a 1 week washout period. In both studies, animals were kindled daily (except during washout period) until they exhibited three consistent stage 5 seizures.

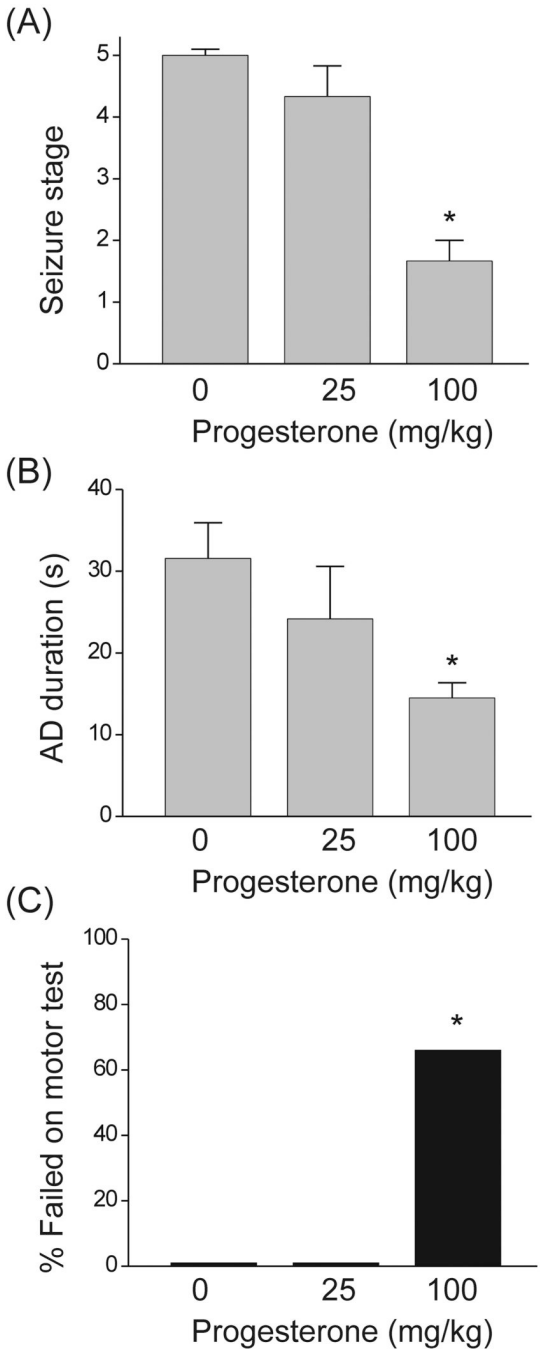

Fig. 2. Effect of P on behavioral seizures (A), afterdischarge duration (B), and motor activity in fully-kindled WT mice

Animals were stimulated daily and were used when they exhibited fully kindled (stage 5) seizures on three consecutive days. P (25 and 100 mg/kg) was injected subcutaneously 30 min before stimulation. The motor effects of P are measured by the inverted screen test. Each bar represents the mean ± SEM of data from six to eight animals. *$p<0.05$ vs. control.

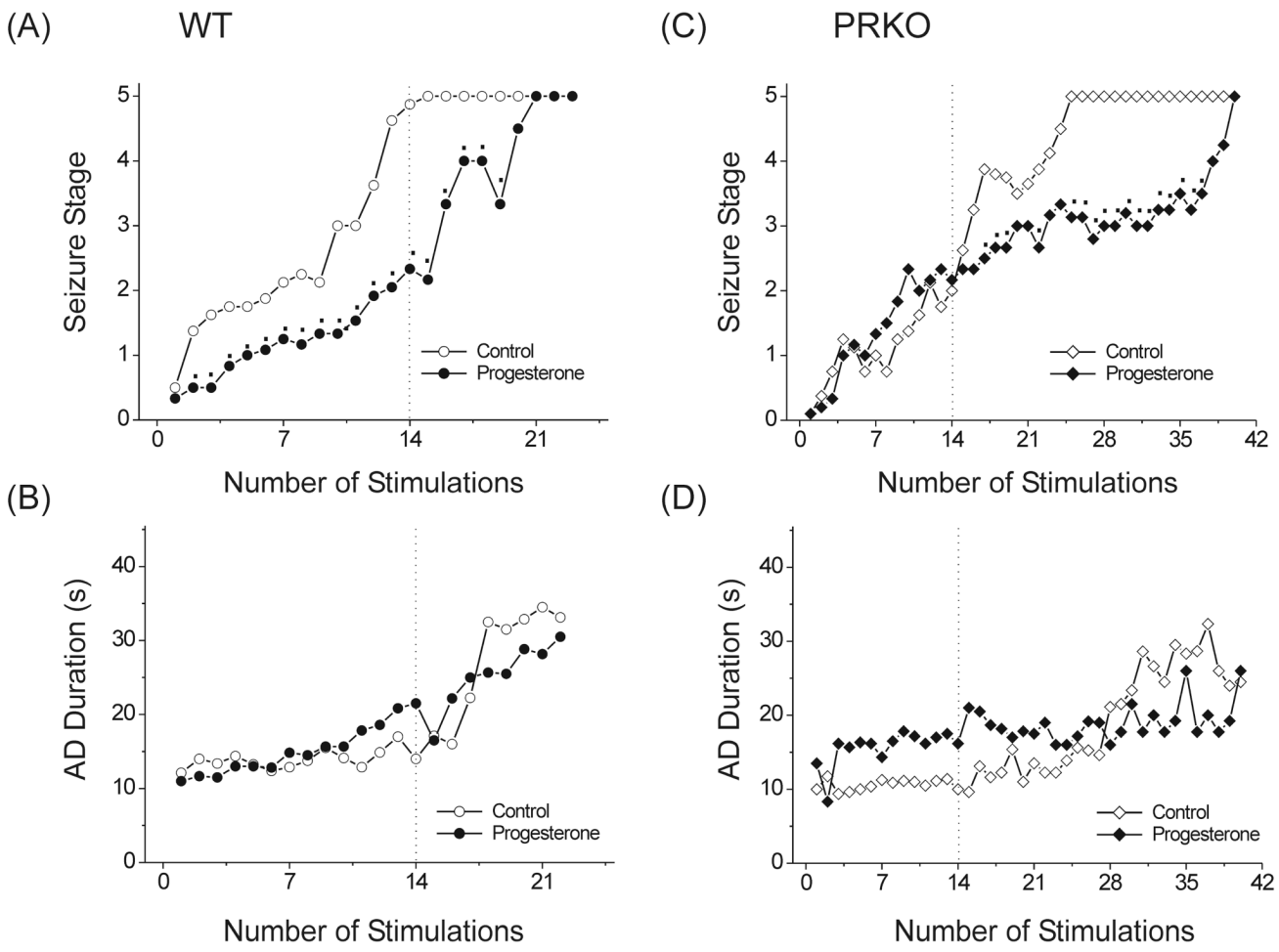

Fig. 3. Inhibition of hippocampus kindling epileptogenesis in WT and PRKO mice by P

P (25 mg/kg, sc) was given twice daily from day 1 to day 14 (dotted line) and animals were stimulated daily (30 min after morning dosing) until they had consistent stage 5 seizures. (A) WT mice treated with P displayed marked retardation of kindling development as expressed by lower mean seizure stage during P treatment and post-drug stimulation sessions. (B) AD duration was similar between control and P groups. (C) PRKO mice treated with P displayed inhibition of kindling development as expressed by lower mean seizure stage during post-drug session. (D) AD duration was similar between control and P groups. Each data point represents the mean of the seizure stage or AD duration of six to eight animals. Small filled squares indicate that the mean value is significantly ($p<0.05$) different from that in control.

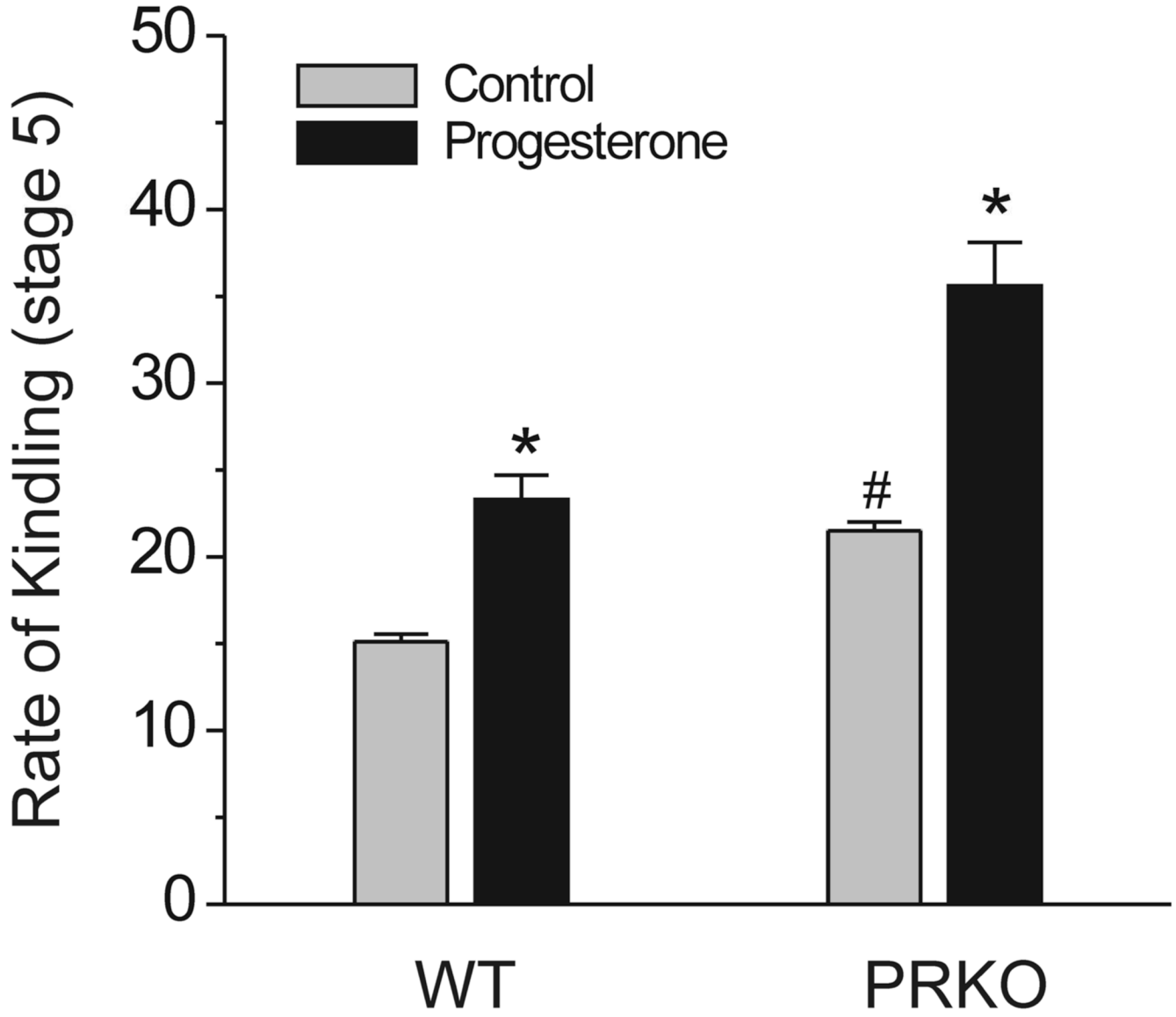

Fig.4. Rate of kindling epileptogenesis in control and P-treated WT and PRKO mice

Rate of kindling (# of stimulations required to elicit third stage 5 seizures) was significantly delayed in P-treated WT and PRKO mice. P (25 mg/kg, sc) was given 30-min prior to stimulation sessions as described in Fig.3. Values represent the mean ± SEM (n = 6–8 mice per group). *p<0.01 vs. control; $^{\#}p$<0.01 vs. control WT mice.

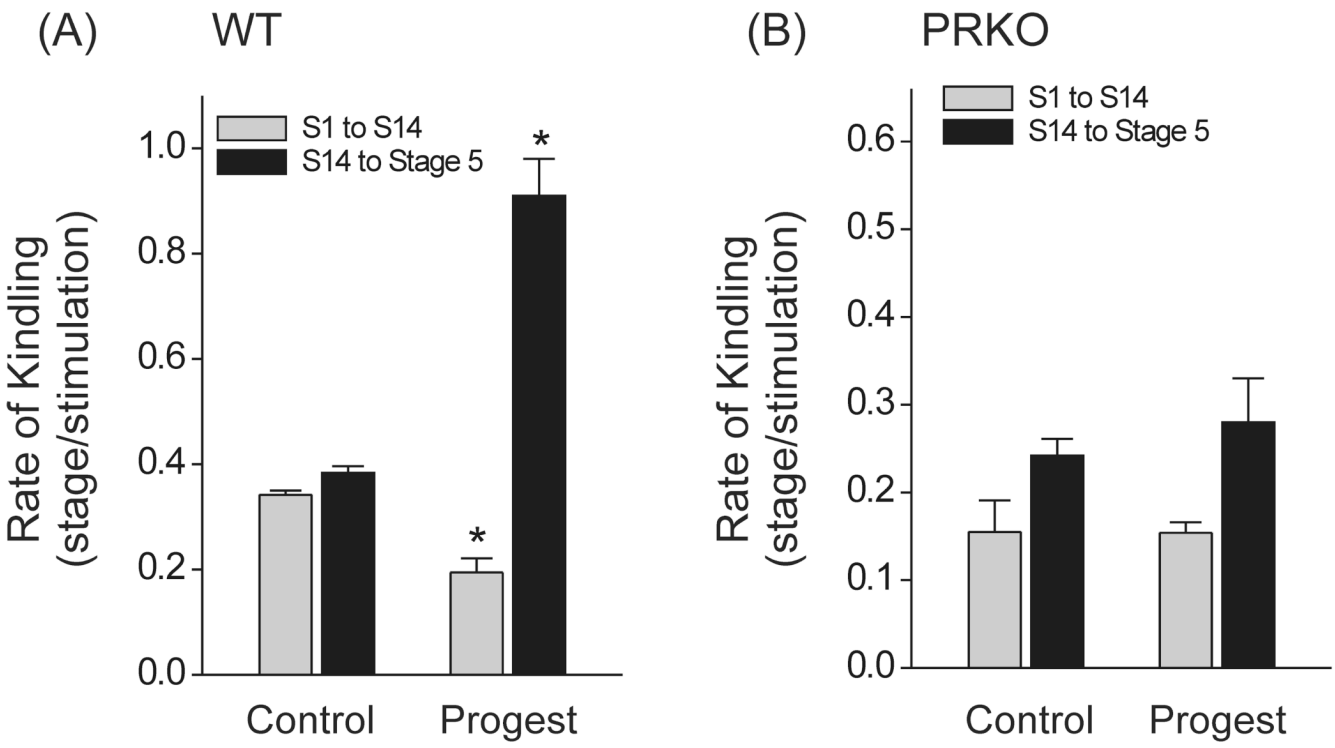

Fig.5. Comparison of the rate of kindling development during P-treatment and P-free periods in WT and PRKO mice

Mean seizure stage values with stimulations 1 through 14 or stimulation 14 through stage 5 kindling (Fig. 3A and 3C) were fit to the linear function $Sn=Rn+A$, where Sn is the mean seizure stage value for the nth stimulation, R is the rate of kindling, and A is set equal to either 0 (stimulations 1 through 14) or S_{14} (stimulations 14 to stage 5 kindling). Correlation coefficients (r) were >0.95 for WT groups. Values represent the mean ± SEM. *p<0.01 vs. control group.

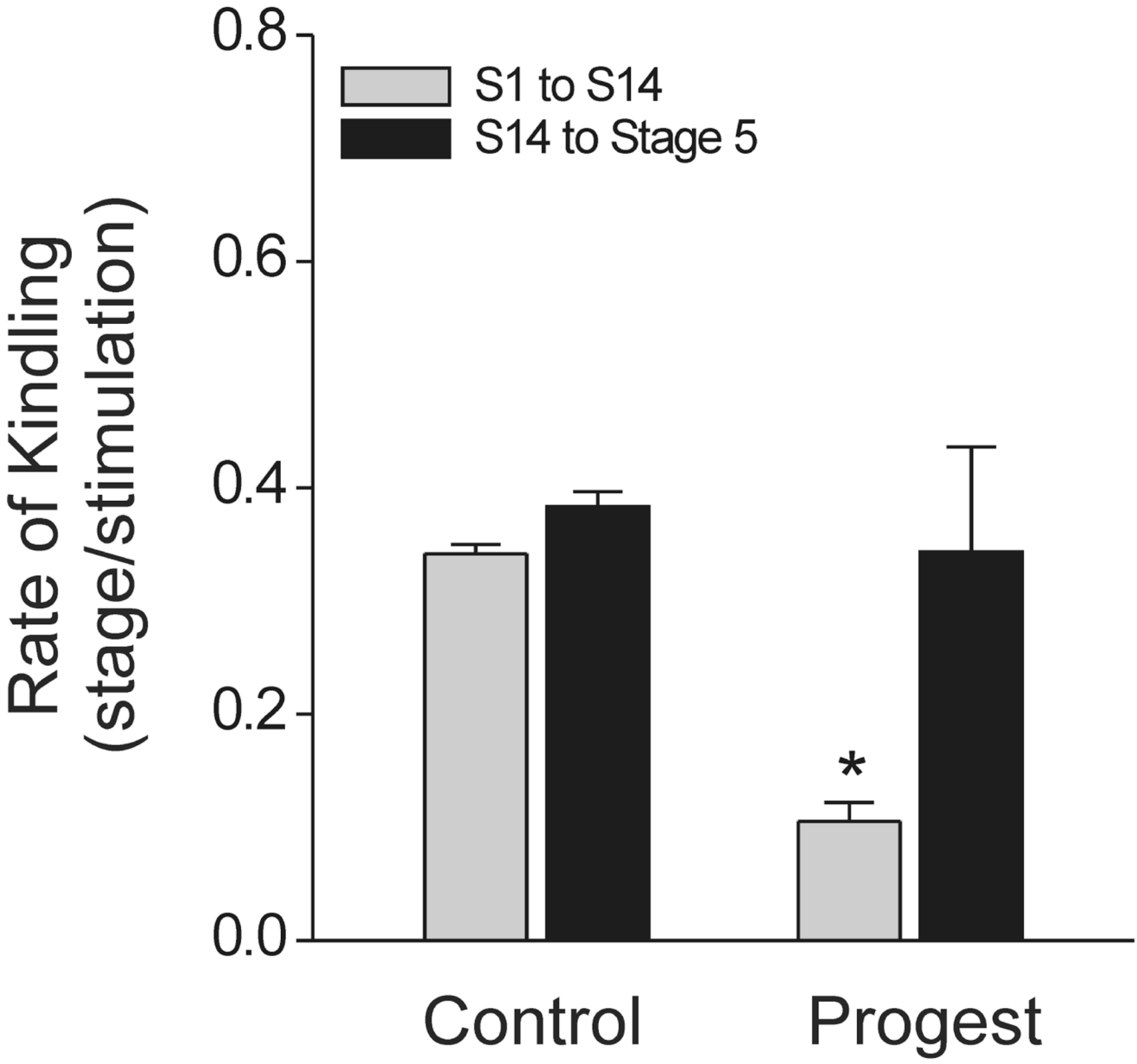

Fig.6. Comparison of the rate of kindling development during P-treatment and following a one week washout period in WT mice

The rate of kindling was calculated as described in Fig.5. Values are shown as the mean ± SEM of six to eight animals.

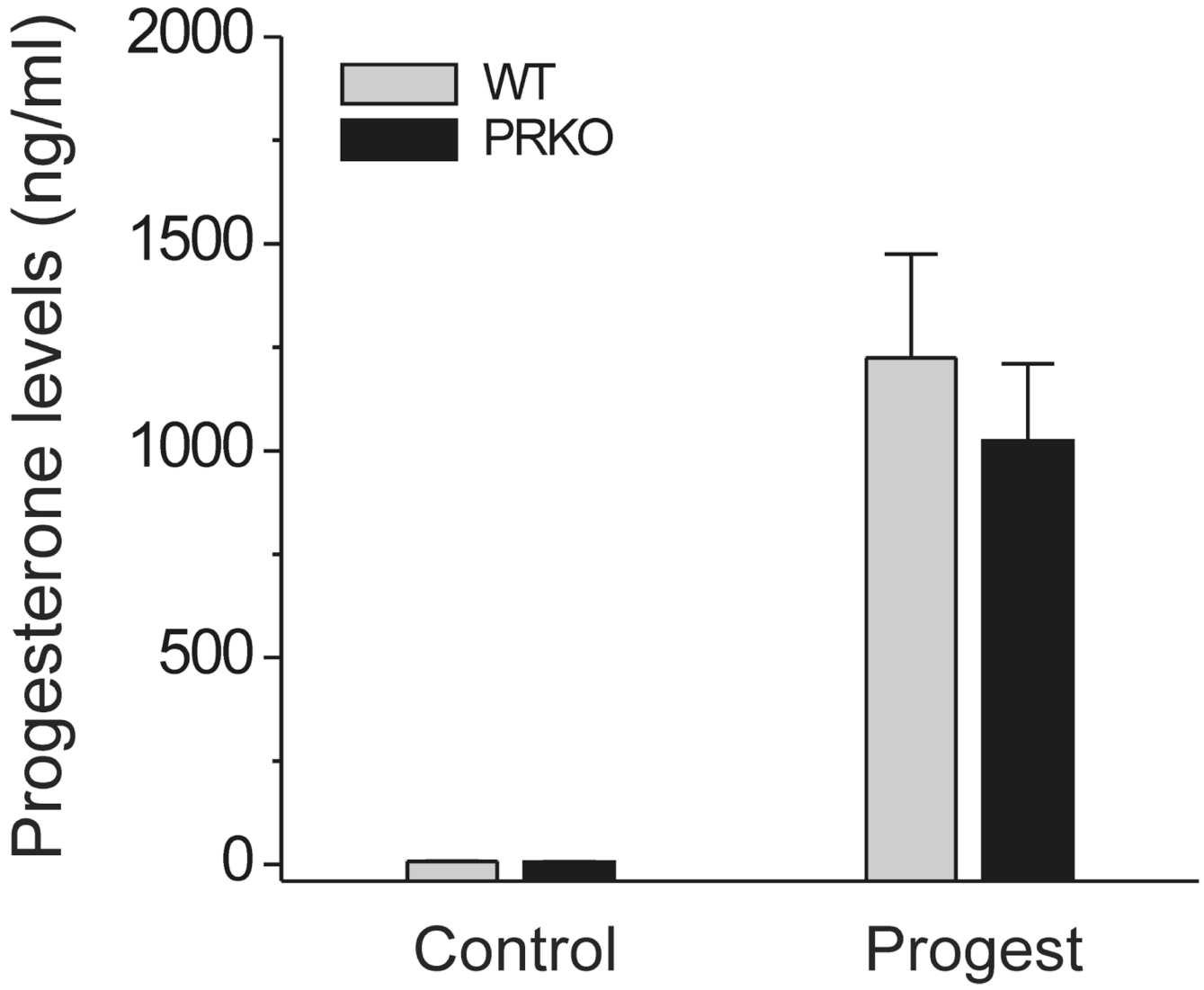

Fig.7. Plasma P levels in control mice and that had been chronically treated with P (25 mg/kg, s.c., twice daily) for 7 days

Plasma samples were taken 30 min after administration of P on the morning after the 7-day chronic treatment period. In control animals, plasma samples were collected 30 min after vehicle injection. Each value represents the mean ± SEM of the levels in four to six animals.

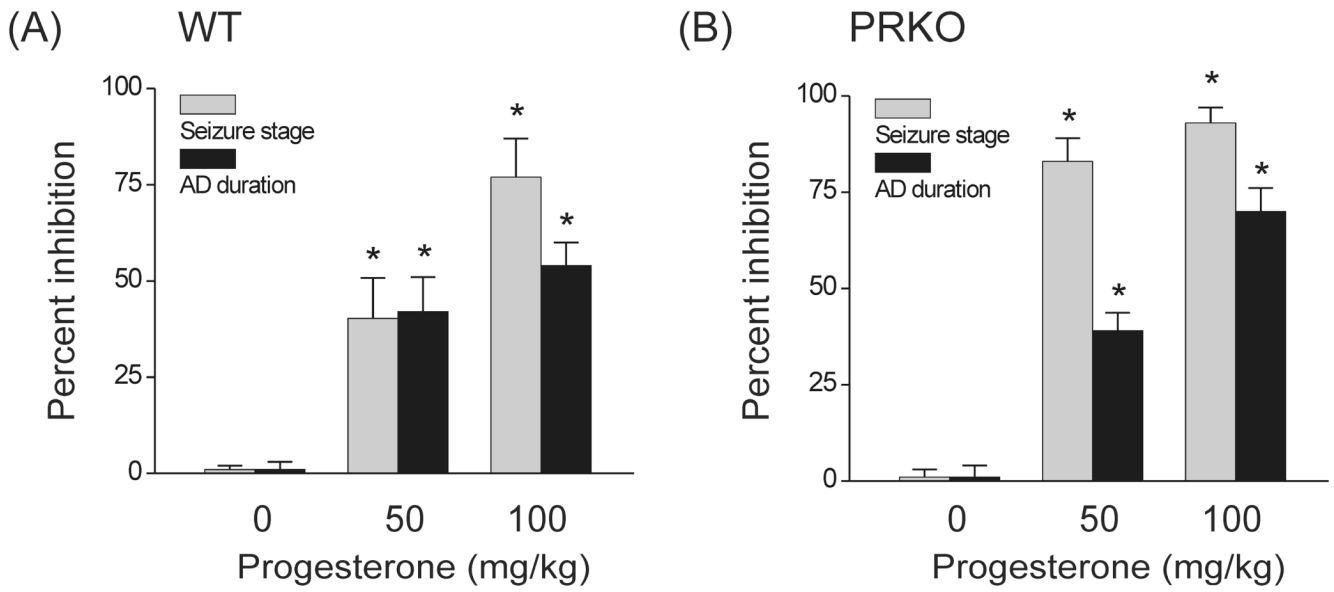

Fig. 8. Inhibition of fully kindled seizures and afterdischarge duration by P in WT (A) and PRKO mice (B)

Animals were stimulated daily and were used when they exhibited fully kindled (stage 5) seizures on three consecutive days. Vehicle or P was injected subcutaneously 30 min before stimulation. Percent inhibition of seizure stage was calculated as $100 \times (1-S/5)$, where S is the seizure stage following drug treatment. Percentage inhibition of afterdischarge was calculated as $100 \times (1-D/D_c)$, where D is the afterdischarge duration after drug treatment and D_c is the average control afterdischarge duration without any drug treatment. The overall mean control AD duration was 31±4 and 26 ±4 s in WT and PRKO mice, respectively. Each bar represents the mean ± SEM (n = 6–8 mice per group). *p<0.01 vs. control.

Table 1

Mean number of stimulations to achieve kindling stages.

Seizure stage	WT		PRKO	
	Control	Progesterone	Control	Progesterone
Stage 1	1.75 ± 0.49	1.34 ± 0.21	3.0 ± 0.27	3.4 ± 0.33
Stage 2	3.38 ± 0.70	9.2 ± 1.77*	10.0 ± 1.64#	9.0 ± 1.03
Stage 3	9.63 ± 0.62	13.5 ± 0.85*	14.0 ± 1.56	18.5 ± 3.06
Stage 4	12.1 ± 0.50	16.5 ± 0.56*	17.7 ± 1.53#	26.2 ± 2.98**
Stage 5	13.2 ± 0.44	21.4 ± 1.38**	19.5 ± 0.5#	34.2 ± 2.68**

Values represent mean ± SEM of number of stimulation values derived from the experiments in Figs. 3A and 3C.

* $p<0.05$;

** $p<0.01$ vs. control;

# $p<0.05$ vs. WT control.