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Involvement of neurosteroids in the anxiolytic-like effects of AC-5216 in mice

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Abstract

AC-5216, a ligand for the translocator protein (18 kDa) (TSPO), previously called the peripheral benzodiazepine receptor (PBR), produces anxiolytic-like effects mediated by TSPO in animal models of anxiety. Since stimulation of TSPO is considered to promote the synthesis of neurosteroids, we investigated the possible role of endogenous neurosteroids that positively act on the $GABA_A$ receptor in the anxiolytic-like effects of AC-5216. In our experiments, the effects of trilostane and finasteride, two inhibitors of steroidogenic enzymes, and picrotoxin, a $GABA_A$ receptor-gated Cl^- channel blocker, on the anxiolytic-like effects of AC-5216 were examined in the social interaction test in mice. Also, the anxiolytic-like effects of allopregnanolone and progesterone were examined. The anxiolytic-like effects of AC-5216 (0.1 mg/kg, p.o.) were inhibited by trilostane (10–30 mg/kg, s.c.), finasteride (10–30 mg/kg, s.c.), and picrotoxin (0.03–0.3 mg/kg, s.c.), while those of diazepam (0.1 mg/kg, p.o.) were inhibited by picrotoxin only. The anxiolytic-like effects of progesterone (1–3 mg/kg, s.c.) were inhibited by finasteride (3–30 mg/kg) and picrotoxin (0.1–0.3 mg/kg), although those of allopregnanolone (10 mg/kg, s.c.) were inhibited by picrotoxin only. These results demonstrate that the anxiolytic-like effects of AC-5216 are due to newly synthesized neurosteroids that enhance $GABA_A$ receptor function.

Keywords: AC-5216; Anxiolytic effect; Translocator protein (18 kDa) (TSPO); Peripheral benzodiazepine receptor (PBR); Mitochondrial benzodiazepine receptor (MBR); Neurosteroid; Trilostane; Finasteride; Picrotoxin; Social interaction; Mice

1. Introduction

The translocator protein (18 kDa) (TSPO) (Papadopoulos et al., 2006), previously called the peripheral benzodiazepine receptor (PBR) or the mitochondrial benzodiazepine receptor (MBR), was initially identified as a peripheral binding site for diazepam, and later distinguished functionally and structurally from the central benzodiazepine receptor (CBR) (Anholt et al., 1984; Beurdeley-Thomas et al., 2000; Casellas et al., 2002). CBR is located on the extracellular domain of $GABA_A$ receptor, and its agonists, such as benzodiazepine anxiolytics, are known to allosterically potentiate the inhibitory action of GABA (Mohler et al., 2002). In contrast, TSPO is located mainly in the outer mitochondrial membrane in peripheral tissues and central nervous system (CNS), and is not linked to the $GABA_A$ receptor (Anholt et al., 1984; Anholt et al., 1986; Basile and Skolnick, 1986). In the CNS, TSPO is mainly located in glial cells (Gallager et al., 1981; Schoemaker et al., 1982) and in neurons (Anholt et al., 1984; Doble et al., 1987). Although the function of TSPO in the CNS remains to be fully disclosed, several lines of evidence have been provided for the involvement of TSPO in synthesis of neurosteroids (Krueger and Papadopoulos, 1990; Papadopoulos et al., 1997). Stimulation of TSPO by appropriate ligands increases the level of neurosteroids (Korneyev et al., 1993; Serra et al., 1999), and it is suggested that this increase is due to TSPO's potential to facilitate the transport of cholesterol from the outer to the inner mitochondrial membrane (Papadopoulos et al., 1997). This transport of cholesterol is known as the rate-limiting step in the synthesis of neurosteroids (Stocco, 2001). In the mitochondria, cholesterol is converted to pregnenolone by $P450_{SCC}$ located in the inner mitochondrial membrane. Pregnenolone moves then to the cytosol where it is processed by several enzymes in a cascade of neurosteroidogenesis. For example, the microsomal enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) converts pregnenolone to progesterone, which is further metabolized to

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allopregnanolone by the microsomal enzymes 5α-reductase and 3α-hydroxysteroid oxidoreductase.

Neurosteroids exert non-genomic effects; they alter neuronal excitability by modulating the activity of several neurotransmitter receptors such as GABA, glutamate and acetylcholine receptors and thus can influence emotion, memory/learning and stress–response (Dubrovsky, 2005; Mellon and Griffin, 2002; Strous et al., 2006). Progesterone-reduced metabolites such as allopregnanolone are known to positively modulate $GABA_A$ receptor function *in vitro* (Gee et al., 1988) and to produce anxiolytic-like effects in several animal models of anxiety (Bitran et al., 1991; Gomez et al., 2002; Picazo and Fernandez-Guasti, 1995; Rodgers and Johnson, 1998; Wieland et al., 1995). These findings suggest that TSPO ligands with anxiolytic effects could exert their action via newly synthesized neurosteroids.

Recently, we have demonstrated that *N*-benzyl-*N*-ethyl-2-(7-methyl-8-oxo-2-phenyl-7,8-dihydro-9*H*-purin-9-yl)acetamide (AC-5216), a novel TSPO ligand synthesized in our laboratories, exhibits high and selective affinity for TSPO derived from rats and human, and that oral administration of AC-5216 produces anxiolytic-like effects in the Vogel-type conflict test in rats, the light/dark box and social interaction tests in mice, and antidepressant-like effects in the forced swimming test in rats (Kita et al., 2004). We have also demonstrated that the anxiolytic-like effects of AC-5216, unlike those of diazepam, are blocked by PK11195, a potent TSPO ligand with presumed *in vivo* antagonistic activity for TSPO, but not affected by flumazenil, a CBR antagonist (Kita et al., 2004). These findings indicate that the anxiolytic-like effects of AC-5216 are mediated by the TSPO.

In the present study, we investigated the possible role of endogenous neurosteroids that positively act on the $GABA_A$ receptor, such as allopregnanolone, in the mechanism underlying the anxiolytic-like effects of AC-5216. In our experiments, the effects of trilostane, a 3β-HSD inhibitor (Potts et al., 1978) and finasteride, a 5α-reductase inhibitor (Rittmaster, 1994; Rittmaster, 1997), and picrotoxin, a $GABA_A$ receptor-gated Cl^- channel blocker, on the anxiolytic-like effects of AC-5216 were evaluated in the social interaction test in mice. To confirm that the action "if any" of these inhibitors on the anxiolytic-like effects of AC-5216 is specific to the mechanism of the action of AC-5216, we evaluated the effects of these inhibitors on the anxiolytic effects of diazepam, which is known to act via CBR, not TSPO (Kita et al., 2004). In addition, although neurosteroids have been reported to exert anxiolytic-like effects in several animal models of anxiety (Bitran et al., 1991; Brot et al., 1997; Picazo and Fernandez-Guasti, 1995; Rodgers and Johnson, 1998), little information on the anxiolytic-like effects of neurosteroids in the social interaction test has been reported (Frye and Rhodes, 2006). Therefore, we evaluated, in this study, the anxiolytic-like effects of allopregnanolone and its precursor progesterone in the social interaction test in mice.

2. Materials and methods

2.1. Animals

Male ddY mice, aged 5 weeks and weighing 22 to 32 g at the time of experiments, were obtained from Japan SLC Inc. (Shizuoka, Japan). In total, 960 mice were used for 17 experiments in this study. Animals were maintained for at least 5 days before experiments in a temperature- and humidity-controlled animal room under a 12:12 h light/dark cycle (light on 06:00 to 18:00) with free access to food and water. They were housed in groups of 5, with the same mates throughout the acclimation and testing period. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Dainippon Sumitomo Pharma Co., Ltd.

2.2. Compounds

AC-5216 was synthesized in our Chemistry Research Laboratories. Allopregnanolone (5α-pregnan-3α-ol-20-one) was purchased from Sigma-Aldrich (SIGMA®, St. Louis, MO, USA), diazepam from Wako Pure Chemical Industries Ltd. (Osaka, Japan), progesterone and picrotoxin from Nacalai Tesque, Inc. (Kyoto, Japan). Trilostane and finasteride were prepared from commercially available formulations in our laboratories. AC-5216 and diazepam were suspended in 0.5% tragacanth gum

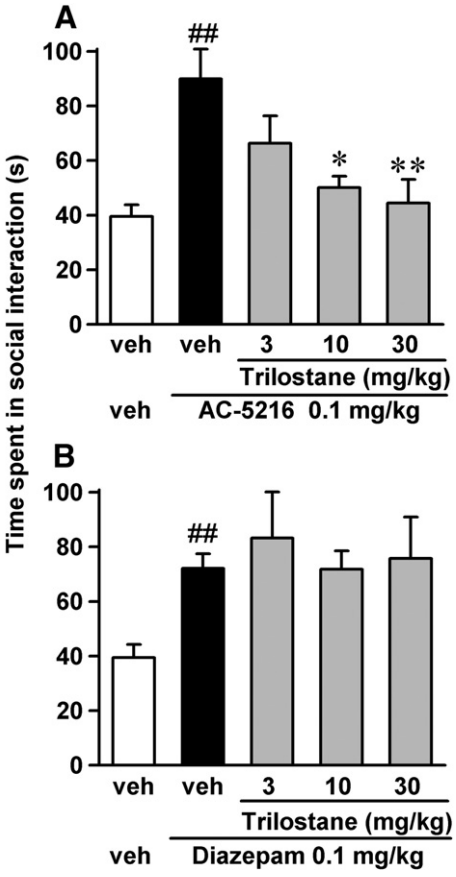

Fig. 1. Effects of trilostane on the anxiolytic-like effects of AC-5216 (A) and diazepam (B) in the social interaction test in mice. Each column represents the mean±S.E.M. of the time spent in social interaction during a 15-min period. $n=5$ pairs. AC-5216 (0.1 mg/kg), diazepam (0.1 mg/kg) or the vehicle (0.5% tragacanth gum solution) was administered orally 1 h before testing. Trilostane or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 1 h before testing. ## $P<0.01$, significantly different from the respective vehicle control group (Student's *t*-test). * $P<0.05$, ** $P<0.01$, significantly different from AC-5216 alone-treated group (Dunnett's test).

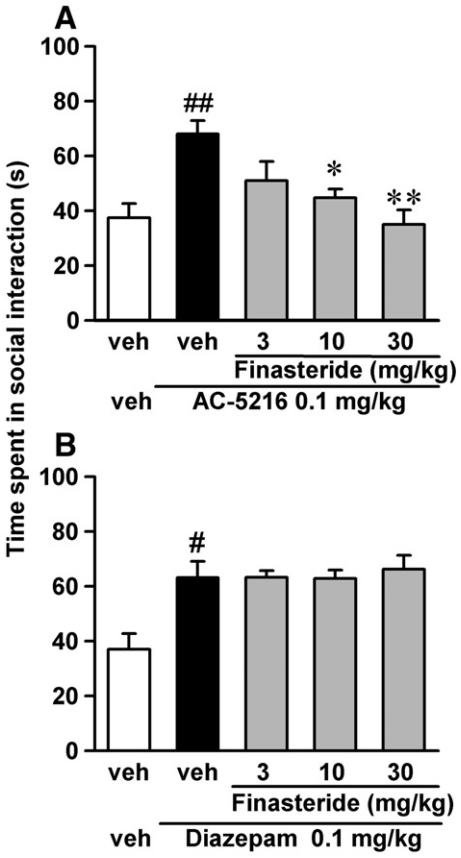

Fig. 2. Effects of finasteride on the anxiolytic-like effects of AC-5216 (A) and diazepam (B) in the social interaction test in mice. Each column represents the mean ± S.E.M. of the time spent in social interaction during a 15-min period. $n=5$ pairs. AC-5216 (0.1 mg/kg), diazepam (0.1 mg/kg) or the vehicle (0.5% tragacanth gum solution) was administered orally 1 h before testing. Finasteride or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 1 h before testing. # $P<0.05$, ## $P<0.01$, significantly different from the respective vehicle control group (Student's t-test). * $P<0.05$, ** $P<0.01$, significantly different from AC-5216 alone-treated group (Dunnett's test).

solution, and orally administered to mice by gavage in a volume of 10 mL/kg. Picrotoxin was dissolved in saline, and trilostane, finasteride, allopregnanolone and progesterone were suspended in saline containing 0.4% Tween 80. These compounds were i.p. or s.c. injected into mice in a volume of 10 mL/kg. In each experiment, all mice received the same number of injections.

2.3. Experimental procedure — social interaction test

The social interaction test was performed according to a previously reported method (Kita et al., 2004). Briefly, experiments were performed under high-light unfamiliar environmental test conditions during the light phase of the light/dark cycle. The apparatus, a glass beaker inverted onto a frosted glass plate, was brightly illuminated with a light source (ca.1200 lx at table level). A video camera was set diagonally above the apparatus and was connected to a monitor placed about 3 m apart from the apparatus. Two mice from separate home cages received the same treatment and were returned to their home cages. At a specific time after treatment, the two mice were placed together in an apparatus, and the time spent in social interaction by the two mice during a 15-min period was manually recorded. Social interaction was defined as sniffing and grooming the partner, genital investigation of the partner, and climbing over or crawling under the partner. After each test, the beaker and plate were washed and wiped. AC-5216 and diazepam were orally administered 1 h before testing at a dose of 0.1 mg/kg. This dose was determined based on a previously conducted study in which both compounds exhibit maximum anxiolytic effects at the indicated dose (Kita et al., 2004). Trilostane and finasteride were administered s.c. 1 h before testing. Picrotoxin, allopregnanolone and progesterone were administered i.p. or s.c. 20 min before testing. The doses of trilostane, finasteride and picrotoxin (3–30 mg/kg for trilostane and finasteride, and 0.03–0.3 mg/kg for picrotoxin) were determined based on preliminary studies in which these compounds, given alone at the indicated doses, had no effect on the time spent in social interaction in mice. On the other hand, the doses of allopregnanolone and progesterone (1–20 mg/kg and 0.1–10 mg/kg) were selected to include doses that have been shown to increase the time spent in social interaction in

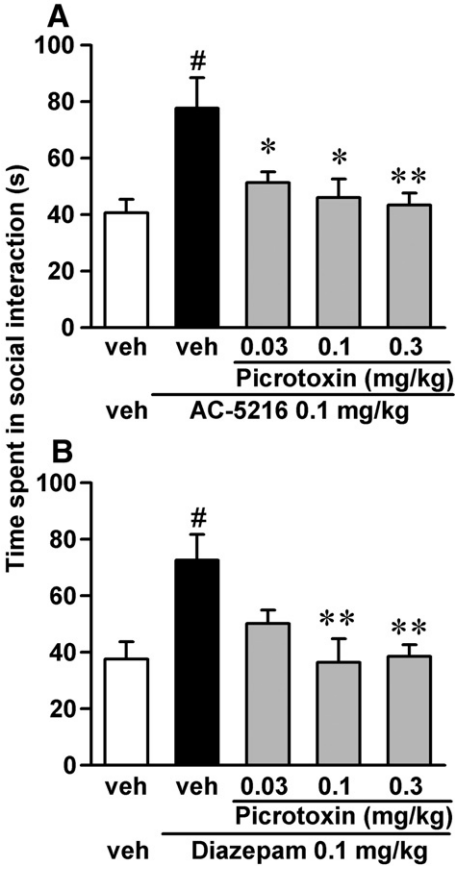

Fig. 3. Effects of picrotoxin on the anxiolytic-like effects of AC-5216 (A) and diazepam (B) in the social interaction test in mice. Each column represents the mean ± S.E.M. of the time spent in social interaction during a 15-min period. $n=5$ pairs. AC-5216 (0.1 mg/kg), diazepam (0.1 mg/kg) or the vehicle (0.5% tragacanth gum solution) was administered orally 1 h before testing. Picrotoxin or the vehicle (saline) was administered s.c. 20 min before testing. # $P<0.05$ significantly different from the respective vehicle control group (Student's t-test). * $P<0.05$, ** $P<0.01$, significantly different from AC-5216 alone- or diazepam alone-treated group (Dunnett's test).

preliminary studies. As in previous studies, the pretreatment time for picrotoxin, allopregnanolone and progesterone was in principle set at 20 min before testing. However, the pretreatment time for trilostane and finasteride was set at 1 h before testing based on a preliminary study showing that trilostane inhibits more potently the effects of AC-5216 when given 1 h before testing.

2.4. Statistical analysis

Data are expressed as mean±S.E.M. of the time spent in social interaction during a 15-min period. In all experiments, except dose–response experiments for allopregnanolone and progesterone, differences in the time spent in social interaction between the vehicle control group and AC-5216-, diazepam-, allopregnanolone- or progesterone-alone treated group were analyzed using Student's *t*-test. When the difference between two groups was statistically significant, differences in the time spent in social interaction between AC-5216-, diazepam-, allopregnanolone- or progesterone-alone treated group and trilostane-, finasteride- or picrotoxin-co-treated group were analyzed using Dunnett's multiple comparison test. In the dose–response experiments for allopregnanolone and progesterone, comparison between the vehicle control group and progesterone- or allopregnanolone-treated group was carried out using Dunnett's multiple comparison test. Values of $P<0.05$ (two-tailed test) were considered significant. Statistical analyses were performed using the SAS® system (SAS Institute Inc., Cary, NC, U.S.A.).

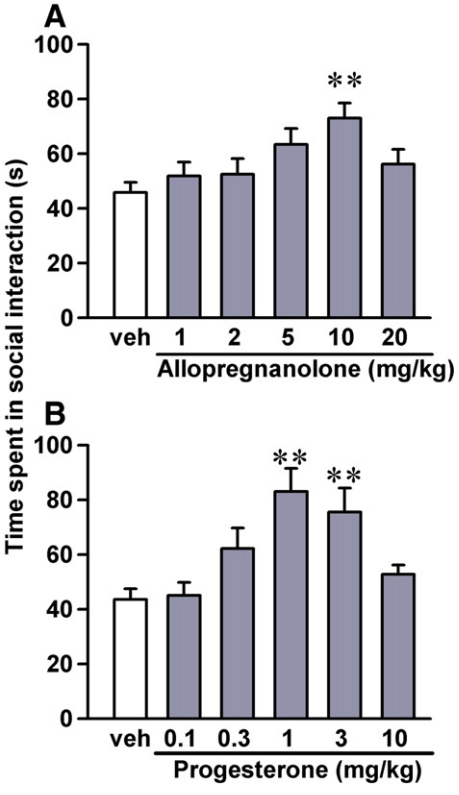

Fig. 4. Effects of allopregnanolone (A) and progesterone (B) on the time spent in social interaction in mice. Each column represents the mean±S.E.M. of the time spent in social interaction during a 15-min period. $n=10$ pairs. Allopregnanolone, progesterone or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 20 min before testing. ** $P<0.01$, significantly different from the respective vehicle control group (Dunnett's test).

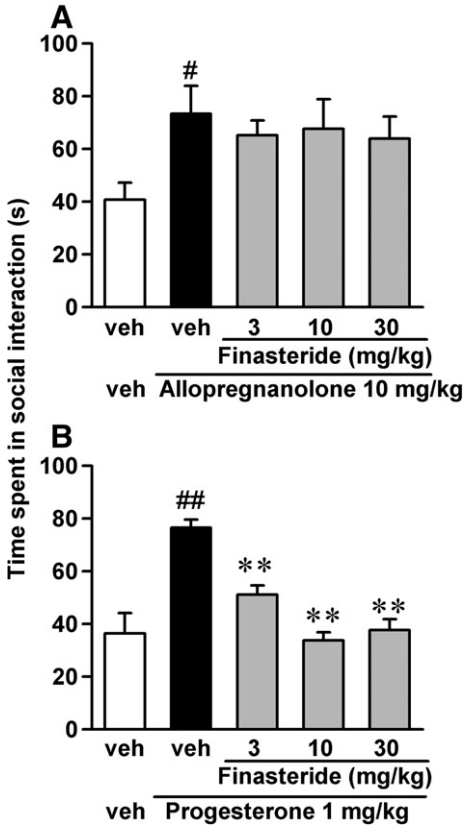

Fig. 5. Effects of finasteride on the anxiolytic-like effects of allopregnanolone (A) and progesterone (B) in the social interaction test in mice. Each column represents the mean±S.E.M. of the time spent in social interaction during a 15-min period. $n=5$ pairs. Allopregnanolone (10 mg/kg), progesterone (1 mg/kg) or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 20 min before testing. Finasteride or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 1 h before testing. # $P<0.05$, ## $P<0.01$, significantly different from the respective vehicle control group (Student's *t*-test). ** $P<0.01$, significantly different from the progesterone-alone treated group (Dunnett's test).

3. Results

3.1. Effects of trilostane on the anxiolytic-like effect of AC-5216

AC-5216 at the dose of 0.1 mg/kg, p.o. significantly increased the time spent in social interaction as compared to the vehicle ($P<0.01$). This increase was significantly inhibited by trilostane at doses of 10 and 30 mg/kg, s.c. ($P<0.05$ and $P<0.01$, respectively) (Fig. 1A). Diazepam at the dose of 0.1 mg/kg, p.o. also induced a significant increase in the time spent in social interaction ($P<0.01$), but the effect of diazepam was not inhibited by trilostane at any of the doses tested (Fig. 1B). Trilostane, when administered alone, had no effect on the time spent in social interaction per se: means±S.E.M. of the time spent in social interaction were 45±4, 46±5, 46±6 and 51±12 (s) for the vehicle-, trilostane 3 mg/kg-, 10 mg/kg- and 30 mg/kg-treated groups, respectively ($n=5$ pairs per group).

3.2. Effects of finasteride on the anxiolytic-like effects of AC-5216

Finasteride at doses of 10 and 30 mg/kg, s.c. significantly inhibited the increase in time spent in social interaction caused by AC-5216 (0.1 mg/kg, p.o.) ($P<0.05$ and $P<0.01$, respectively) (Fig. 2A), but not that caused by diazepam (0.1 mg/kg, p.o.) (Fig. 2B). Finasteride, when administered alone, had no effect on the time spent in social interaction per se: means±S.E.M. of the time spent in social interaction were 41±8, 47±10, 42±7 and 40±7 (s) for the vehicle-, finasteride 3 mg/kg-, 10 mg/kg- and 30 mg/kg-treated groups, respectively ($n=5$ pairs per group).

3.3. Effects of picrotoxin on the anxiolytic-like effects of AC-5216

Picrotoxin at doses of 0.03, 0.1 and 0.3 mg/kg, s.c. significantly inhibited AC-5216-induced increase in time spent in social interaction ($P<0.05$, $P<0.05$ and $P<0.01$, respectively) (Fig. 3A). Similarly, picrotoxin at doses of 0.1 and 0.3 mg/kg, s.c. inhibited diazepam-induced increase in time spent in social interaction ($P<0.01$) (Fig. 3B). Picrotoxin, given alone at any of the doses tested, did not affect the time spent in social interaction per se: means±S.E.M. of the time spent in social interaction were 45±3, 48±7, 46±7 and 48±6 (s) for the vehicle-, picrotoxin 0.03 mg/kg-, 0.1 mg/kg- and 0.3 mg/kg-treated groups, respectively ($n=5$ pairs per group).

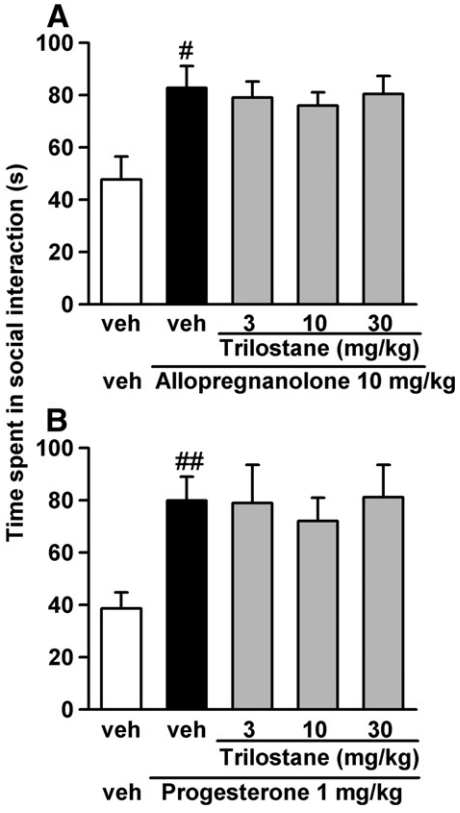

Fig. 6. Effects of trilostane on the anxiolytic-like effects of allopregnanolone (A) and progesterone (B) in the social interaction test in mice. Each column represents the mean±S.E.M. of the time spent in social interaction during a 15-min period. $n=5$ pairs. Allopregnanolone (10 mg/kg), progesterone (1 mg/kg) or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 20 min before testing. Trilostane or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 1 h before testing. # $P<0.05$, ## $P<0.01$, significantly different from the respective vehicle control group (Student's t-test).

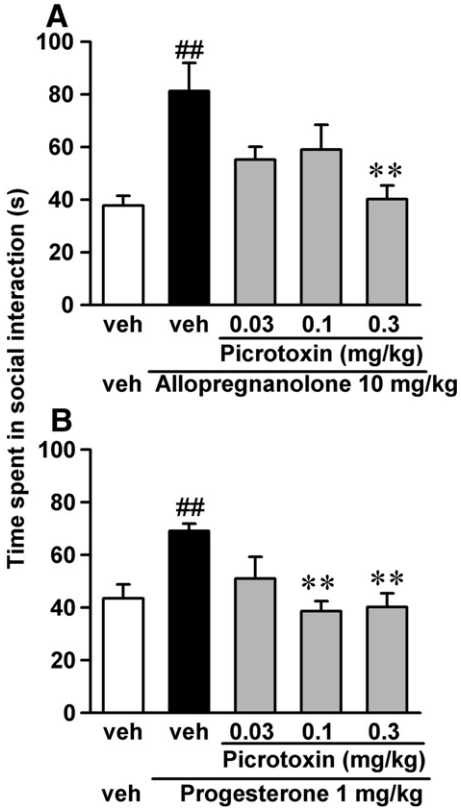

Fig. 7. Effects of picrotoxin on the anxiolytic-like effects of allopregnanolone (A) and progesterone (B) in the social interaction test in mice. Each column represents the mean±S.E.M. of the time spent in social interaction during a 15-min period. $n=5$ pairs. Allopregnanolone (10 mg/kg), progesterone (1 mg/kg) or the vehicle (saline containing 0.4% Tween 80) was administered s.c. 20 min before testing. Picrotoxin or the vehicle (saline) was administered i.p. 20 min before testing. ## $P<0.01$, significantly different from the respective vehicle control group (Student's t-test). ** $P<0.01$, significantly different from allopregnanolone alone- or progesterone-alone treated group (Dunnett's test).

3.4. Anxiolytic-like effects of neurosteroids in social interaction test in mice

Allopregnanolone at the dose of 10 mg/kg, s.c. significantly increased the time spent in social interaction ($P<0.01$), although this increase was only slight at the lower dose of 5 mg/kg (Fig. 4A). At the highest dose of 20 mg/kg, the anxiolytic-like effect of allopregnanolone decreased. Progesterone at doses of 1 and 3 mg/kg, s.c. significantly increased the time spent in social interaction ($P<0.01$) (Fig. 4B) and this effect decreased at the highest dose of 10 mg/kg.

3.5. Effects of finasteride, trilostane and picrotoxin, on the anxiolytic-like effects of allopregnanolone and progesterone

Finasteride at doses of 3–30 mg/kg significantly inhibited progesterone (1 mg/kg)-induced increase in time spent in social interaction ($P<0.01$), but not allopregnanolone (10 mg/kg)-induced increase in time spent in social interaction (Fig. 5A, B).

On the other hand, trilostane at doses of 3–30 mg/kg had no effect on either allopregnanolone (10 mg/kg)- or progesterone (1 mg/kg)-induced increase in time spent in social interaction (Fig. 6A, B). Picrotoxin at 0.3 mg/kg inhibited allopregnanolone-induced increase in time spent in social interaction ($P<0.01$) (Fig. 7A). In addition, picrotoxin at 0.1 and 0.3 mg/kg inhibited progesterone-induced increase in time spent in social interaction ($P<0.01$) (Fig. 7B).

4. Discussion

In the present study, the possible involvement of endogenous neurosteroids in the anxiolytic-like effects of AC-5216 was investigated using two inhibitors of key steroidogenic enzymes, i.e. trilostane and finasteride, and the $GABA_A$ receptor-gated Cl^- channel blocker picrotoxin in the social interaction test in mice. Our results show that newly synthesized neurosteroids that enhance $GABA_A$ receptor function are involved in the anxiolytic-like effects of AC-5216. Indeed, we found that trilostane, finasteride and picrotoxin inhibited the anxiolytic-like effects of AC-5216.

The finding that the anxiolytic-like effects of AC-5216 were inhibited by trilostane suggests that neurosteroids synthesized via 3β-HSD, such as progesterone or its metabolites, are involved in the mechanism of action of AC-5216. Furthermore, inhibition by finasteride of AC-5216 anxiolytic-like effects suggests that metabolites of progesterone rather than progesterone itself are involved in the anxiolytic-like effects of AC-5216. This finding is in line with a previous finding showing that the anxiolytic-like effects of FGIN1-27, a potent TSPO ligand, are inhibited by 4-MA, a 5α-reductase inhibitor (Bitran et al., 2000). The specificity of the two steroidogenic enzyme inhibitors used in this study (trilostane and finasteride) is supported by the study of Phan et al. (1999), which demonstrated that trilostane and finasteride inhibit their respective target enzymes in the mouse hippocampus even after repeated administration. In addition, since these two steroidogenic enzyme inhibitors, when administered alone, had no effect on the time spent in social interaction in mice, it is considered that the inhibition by trilostane and finasteride of AC-5216 anxiolytic-like effects is due to specific behavioral suppression. These findings strongly suggest that AC-5216 exerts its anxiolytic-like effects through the action of newly synthesized endogenous neurosteroids, especially progesterone-reduced metabolites. The anxiolytic-like effects of AC-5216 were also inhibited by picrotoxin, indicating that the effects of AC-5216 are mediated by enhancement of $GABA_A$ receptor-gated Cl^- conductance. This result is consistent with the results of previous studies showing that the anxiolytic-like effects of FGIN1-27 are inhibited by 4-MA and PK11195, and picrotoxin (Bitran et al., 2000), and those of Ro5-4864 are inhibited by PK11195 (Reddy and Kulkarni, 1996) and picrotoxin (Reddy and Kulkarni, 1997). We have previously found in *in vitro* binding studies that AC-5216 has no affinity for the GABA binding site, the picrotoxin binding site or the CBR, and that PK11195 completely inhibits AC-5216-induced anxiolytic-like effects (Kita et al., 2004). These findings indicate that AC-5216 effects are due to indirect enhancement of $GABA_A$ receptor function. Collectively, the evidence presented above support the hypothesis that AC-5216, upon binding to the TSPO, stimulates the synthesis of neurosteroids de novo, especially ring A-reduced metabolites of progesterone, such as allopregnanolone, which potentiate $GABA_A$ receptor function, and consequently exerts its anxiolytic-like effect.

In contrast to inhibition of AC-5216 anxiolytic-like effects, trilostane and finasteride did not affect diazepam anxiolytic action in this study. Diazepam has been shown to bind to TSPO as well as CBR with similar affinity in *in vitro* binding studies (Kita et al., 2004), and to stimulate the synthesis of pregnenolone in brain mitochondria (McCauley et al., 1995). However, diazepam anxiolytic effects in rats and mice are blocked by flumazenil, but not by PK 11195 (Kita et al., 2004; Przegalinski et al., 2000; Romeo et al., 1992), suggesting that the anxiolytic effects of diazepam are mediated by CBR, but not by TSPO. These findings together with our results in this study indicate that newly synthesized neurosteroids are not involved in the anxiolytic effects of diazepam, and confirm that diazepam anxiolytic effects, which were blocked by picrotoxin in this study, are mainly due to CBR-mediated potentiation of $GABA_A$ receptor function.

Regarding the inhibition of AC-5216-produced effects by trilostane and finasteride, it is important to mention that these inhibitors act on various metabolic pathways of steroidogenesis, although their specific inhibitory activity is for 3β-HSD and 5α-reductase, respectively. Trilostane can block the conversion of not only pregnenolone to progesterone, but also that of dehydroepiandrosterone to androstenendione. Finasteride, on the other hand, can block the conversion of progesterone to 5α-dihydroprogesterone, 11-deoxycorticosterone to 5α-dihydrodeoxycorticosterone, and testosterone to dihydrotestosterone. Therefore, the inhibition of AC-5216-produced effects by trilostane and finasteride may be due to blockade of various pathways. However, the finding that both trilostane and finasteride completely inhibited the anxiolytic-like effects of AC-5216 suggests that neurosteroids synthesized via pathways including both 3β-HSD and 5α-reductase are involved in the mechanism of action of AC-5216. In addition, it should be noted that inhibition of neurosteroid synthesis may lead to accumulation of precursor steroids, or other species, involved in the enzymatic reaction rather than decrease in products. Accumulated precursor steroids, which may be anxiogenic substances, may inhibit the anxiolytic-like effects of AC-5216. To clarify these points, further experiments to evaluate the effects of precursor steroids on AC-5216 anxiolytic-like effects are needed. On the other hand, the finding in this study that finasteride did not affect the time spent in social interaction per se is inconsistent with previously reported results showing that finasteride increases social interaction in proestrous female rats (Rhodes and Frye, 2001). These contrasting results may be due to the sex difference or stage of the estrous cycle, or the possibility that social interaction in proestrous female rats, unlike male rats, reflects exploration rather than anxiety. It is therefore important to consider the influence of steroidogenic enzyme inhibitors while taking into consideration the sex of the animals tested and the stage of estrous cycle when the tested animals are females.

In our experiments, both allopregnanolone and progesterone produced anxiolytic-like effects in the social interaction test in mice with an inverted U-shaped dose–response curve. This dose–response profile is consistent with previously reported results of behavioral effects of these steroids on anxiety (Rodgers and Johnson, 1998; Gomez et al., 2002) and mood (Andréen et al., 2006). Regarding the mechanism underlying the anxiolytic effects of allopregnanolone and progesterone, it is important to consider the fact that the effects of these steroids were inhibited by picrotoxin. Progesterone, unlike allopregnanolone, has been shown to have no enhancing action on $GABA_A$ receptor function in *in vitro* assays (Gee et al., 1987), although its effects in behavioral assays have been found to be related to enhancement of GABAergic function. These findings suggest that the effects of progesterone observed in behavioral assays are due to its metabolites. This idea may be supported by our finding that finasteride inhibited progesterone anxiolytic-like effects and by previous studies showing that 4-MA, a 5α-reductase inhibitor, inhibits progesterone anxiolytic-like effects (Bitran et al., 1995). Allopregnanolone, on the other hand, is known to directly interact with $GABA_A$ receptor at a neurosteroid recognition site, different from CBR (Lambert et al., 1995). This finding is supported by the results of behavioral studies showing that the anxiolytic-like effects of allopregnanolone are not affected by flumazenil, a CBR antagonist (Brot et al., 1997), and by our results in a preliminary study showing that flumazenil (10 mg/kg, i.p.) had no effect on either allopregnanolone (10 mg/kg, s.c.)- or progesterone (1 mg/kg, s.c.)-induced anxiolytic-like effects in the social interaction test in mice (data not shown). Regarding the anxiolytic-like effects of progesterone, it should be noted that in most previous studies, progesterone was administered 4 h before testing (Bitran et al., 1993; Picazo and Fernandez-Guasti, 1995) because it had been considered that a time lag is necessary for the conversion of progesterone to its reduced metabolites in the body. On the other hand, in the present study, progesterone, administered 20 min before testing, could produce anxiolytic-like effects in mice. In addition, it has been reported that progesterone administered 30 min before testing can inhibit pentylentetrazol-induced convulsion in mice and that this effect is completely blocked by finasteride (Kokate et al., 1999). More recently, Gomez et al. (2002) showed that progesterone produces anxiolytic-like effects as early as 30 min after i.p. administration in rats. Taken together, these findings indicate that progesterone might be rapidly converted to its metabolites, which exert pharmacological effects related to enhancement of $GABA_A$ receptor function. The finding that both picrotoxin and finasteride inhibited progesterone and AC-5216 anxiolytic-like effects, further supports the hypothesis that endogenous progesterone-reduced metabolites synthesized de novo are involved in the anxiolytic-like effects of AC-5216.

The results of the present study indicate the involvement of endogenous neurosteroids in the anxiolytic-like effects of AC-5216, but could not clarify the origin of these steroids. Therefore, it is wiser to use the term "neuroactive steroids" instead of "neurosteroids", which only means steroids synthesized in the nervous system. To further support the relationship between neurosteroids and the anxiolytic-like effects of AC-5216, we investigated in a separate supplementary study the effects of AC-5216 on steroids levels in the brain. AC-5216 was found to increase allopregnanolone level in the brain of rats (data not shown), although no supportive evidence for this finding was obtained from *in vitro* assays. Further studies are needed to confirm the effects of AC-5216 on steroid levels in the brain.

Neurosteroids are involved in various CNS functions; emotion, memory/learning, and stress–response (Dubrovsky, 2005; Mellon and Griffin, 2002; Strous et al., 2006) and social behavior (Frye, 2001). Since social behavior is reported to be affected by endogenous steroid levels (Frye et al., 2000), it may be possible that the anxiolytic-like effects of AC-5216 in the social interaction test do not reflect anxiolysis as increased social behavior. However, this is unlikely because AC-5216 has been found to produce anxiolytic-like effects not only in the social interaction test but also in different paradigms such as the rat Vogel-type conflict test and the mouse light/dark box test (Kita et al., 2004). In addition, AC-5216 does not affect the time spent in social interaction under less aversive environmental conditions (low-light and familiarity) or spontaneous locomotor activity in mice (data not shown). These findings suggest that AC-5216-induced increase in time spent in social interaction is not due to increased social activity or locomotor activity. Therefore, it is believed that the effects of AC-5216 observed in the social interaction test reflect anxiolysis.

In conclusion, the results of the present study demonstrate that the anxiolytic-like effects of AC-5216 are due to newly synthesized neurosteroids that enhance $GABA_A$ receptor function.

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