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The anxiolytic-like effects of the neurosteroid allopregnanolone: interactions with $GABA_A$ receptors

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Abstract

The neurosteroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) was administered systemically to rats which were tested in the Geller-Seifter conflict paradigm, an established animal model of anxiety. Allopregnanolone was found to produce significant anxiolytic-like effects at a dose of 8 mg/kg. When three ligands that function at different sites on the γ-aminobutyric acid/benzodiazepine receptor-chloride ionophore complex ($GABA_A$ receptors) were examined in conjunction with allopregnanolone, the anti-conflict effects of allopregnanolone were effectively reversed only by the benzodiazepine receptor inverse agonist RO15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5-α]-[1,4]benzodiazepine-3-carboxylate). Since this inverse agonist has been reported to inhibit the $GABA_A$-activated chloride flux in neuronal membranes, it is likely that the stimulation of the chloride channel in $GABA_A$ receptors is an important component of the effects of allopregnanolone. In contrast, the benzodiazepine receptor antagonist flumazenil (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5-α]-[1,4]benzodiazepine-3-carboxylate) did not block the anxiolytic-like actions of allopregnanolone, indicating that allopregnanolone does not bind at the benzodiazepine site directly. Isopropylbicyclophosphate, which binds at the picrotoxinin site on the $GABA_A$ receptors and blocks the behavioral actions of ethanol, also dose-dependently reversed the anti-conflict effect of this neurosteroid. The results suggest that allopregnanolone may be working either at a site specific for the benzodiazepine receptor inverse agonist RO15-4513 or at the picrotoxinin site to produce its potent anxiolytic-like behavioral effects.

Keywords: Anxiety; Conflict; Neurosteroid; 3α-Hydroxy-5α-pregnan-20-one; $GABA_A$ receptor; Benzodiazepine

1. Introduction

Neurosteroids have been shown to alter rapidly the excitability of the central nervous system, producing behavioral effects through non-genomic mechanisms within seconds to minutes (Paul and Purdy, 1992; Olsen and Sapp, 1995; Rabow et al., 1995). As initially demonstrated by Baulieu (1991), neurosteroids including 3α-hydroxy-5α-pregnan-20-one or allopregnanolone can be biosynthesized in the mammalian nervous system (Akwa et al., 1991; Robel and Baulieu, 1994). In addition, allopregnanolone can be secreted by the ovary and adrenal gland (Holzbauer, 1976; Holzbauer et al., 1985). Allopregnanolone and 3α-hydroxysteroids of related structure have been shown to be potent anxiolytic, anticonvulsant, sedative/hypnotic and anesthetic agents through their allosteric modulation of the γ-aminobutyric acid/benzodiazepine receptor- chloride ionophore complex ($GABA_A$ receptors) (Harrison and Simmonds, 1984; Lambert et al., 1987; Simmonds, 1991; Paul and Purdy, 1992; Olsen and Sapp, 1995; Rabow et al., 1995). Allopregnanolone and its synthetic 11-keto derivative, 3α-hydroxy-5α-pregnan-11,20-dione (alphaxalone) increase responding in the conflict portion of the Geller-Seifter test and produce similar anxi-

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olytic-like effects in the elevated plus-maze test (Britton et al., 1991; Wieland et al., 1995). The mechanism by which allopregnanolone and related steroids modulate $GABA_A$ receptors is believed to be distinct from that of barbiturates and benzodiazepines based on evidence from electrophysiological and neurochemical studies (Majewska et al., 1986; Gee et al., 1988; Peters et al., 1988; Puia et al., 1992; Lan et al., 1991; Morrow et al., 1990).

Many anxiolytic drugs exert their behavioral effects by binding to the benzodiazepine site on $GABA_A$ receptors. There is evidence that a steroid binding site exists on this receptor complex as well (Morrow et al., 1990; Lan et al., 1991). When allopregnanolone binds to $GABA_A$ receptors, it causes enhanced GABA and benzodiazepine binding to neuronal membranes resulting in increased GABA-induced chloride transport and higher $GABA_A$ receptor-mediated neuronal currents. Thus, allopregnanolone may produce psychopharmacological effects similar to those of classic anxiety-reducing agents by modulating the chloride channel of $GABA_A$ receptors.

The purpose of the present study was to examine the anxiolytic-like effects of allopregnanolone in ovariectomized female rats using the Geller-Seifter modified conflict test, an animal model of anxiety that is particularly sensitive to the $GABA_A$ receptor ligands. Female rats were used because allopregnanolone is a metabolite of progesterone, and there is evidence of differential sensitivity of female animals to allopregnanolone (Grant et al., 1996; Azarov et al., 1997). Experiments were conducted to investigate the neuropharmacological mechanism of action of allopregnanolone by administering it in conjunction with three ligands that have been hypothesized to bind at different sites on $GABA_A$ receptors: flumazenil, a benzodiazepine receptor antagonist which reverses the anti-conflict effects of benzodiazepines (Koob et al., 1986), RO15-4513, a benzodiazepine receptor partial inverse agonist that counteracts several effects of alcohol (Suzdak et al., 1986; Britton et al., 1988; Lister, 1987), and isopropylbicyclophosphate, a potent ligand at the picrotoxinin binding site which reverses the anti-conflict effect of ethanol (Koob et al., 1989) were administered. The purpose of this study was to measure the effectiveness of these compounds in reversing the behavioral effects of allopregnanolone in the conflict test, thereby gaining insight into the site(s) of action of allopregnanolone within the $GABA_A$ receptor complex.

2. Materials and methods

2.1. Animals

Sixty female albino Wistar rats (Charles River) weighing approximately 350 g at the time of the experiments were group-housed in polyethylene cages with sawdust bedding. The colony room was on a 12:12 light/dark cycle (6 a.m.) and all experiments were performed in the morning. The rats were trained on the modified Geller-Seifter operant test of anxiety for several weeks prior to the start of the study, during which time they were maintained at about 85% of their free-feeding weight with 15 g food per day in addition to that received as a reward (approximately 2 g) during the testing paradigm. This feeding schedule continued through the testing phase of the study.

2.2. Surgery

During the 2 weeks that they were being trained to press on the operant test, rats were ovariectomized under halothane anesthesia to eliminate the influence of fluctuations in ovarian hormones with the estrous cycle. Rats recovered by the next day and resumed operant training at that time.

2.3. Conflict test

Rats were first trained to lever-press for 45 mg Noyes food pellets on a continuous reinforcement schedule in sound-attenuated operant chambers (Coulbourn Instruments, Lehigh Valley, PA, USA) equipped with stainless steel bars on the floor through which electric shocks could be delivered. Once they learned to press the lever consistently (averaging about 40 presses/min), the rats were switched to a random interval 30 s reinforcement schedule and then were trained on a multiple-schedule conflict test. When they achieved stable responding (defined as $\pm 15\%$ of the mean for 3 successive days) on both the unpunished and punished sections, the testing began.

The conflict test consisted of three components: a pure reward (unpunished) component, a time-out component, and a conflict (punished) component. Responses made during the reward component were reinforced on a random interval 30 s schedule in a darkened chamber. The chamber was illuminated with a house light during the time-out component, and there was no reinforcement provided. The third component (conflict) was signaled by a light flashing above the lever. Responses during the conflict component were rewarded with food and simultaneously punished with footshocks on a continuous reinforcement schedule. The footshocks consisted of a scrambled, biphasic square-wave produced by a SGS-003 simulator (BRS/LVE Division of Tech. Serv., Laurel, MD, USA). The current was increased by 0.15 mA with each successive shock during the conflict component up to a maximum of 3.3 mA (equivalent to approximately 0.8 mA, 60 Hz sine wave). The testing session was composed of two cycles of 9 min each, including a 5 min unpunished component, a 2 min time-out component, and a 2 min punished component.

2.4. Drugs and treatments

Allopregnanolone was synthesized and purified as described previously (Purdy et al., 1992). It was suspended in a 4 mg/ml solution of 20% 2-hydroxypropyl-β-cyclodextrin (β-cyclodextrin, Aldrich, Milwaukee, WI, USA) and saline by rapid sonication with a probe sonicator. Allopregnanolone was administered s.c. at a dose of 4 or 8 mg/kg in the first study and at a dose of 8 mg/kg in the subsequent studies. The vehicle alone was administered at a dose of 4 mg/kg. All doses were injected 30 min prior to the conflict testing.

Flumazenil (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5-α]-[1,4]benzodiazepine-3-carboxylate), gift from Hoffmann-La Roche, Nutley, NJ, USA) was dissolved in a solution of 50% saline, 40% propylene glycol, and 10% ethanol, and injected i.p. in doses of 0 (vehicle), 1.5, 3, and 6 mg/kg, 30 min prior to conflict testing.

RO15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5-α]-[1,4]benzodiazepine-3-carboxylate, gift from Hoffmann-La Roche, Nutley, NJ, USA) was dissolved in a solution of 95% saline and 5% ethanol, and injected i.p. in doses of 0 (vehicle), 0.75 or 1.5 mg/kg, 20 min prior to conflict testing.

Isopropylcyclophosphate (generously provided by Dr Richard Squires) was dissolved in saline and injected i.p. at doses of 0 (saline), 5, 10 and 15 mg/kg, 20 min prior to conflict testing.

For the drug interaction experiment, each treatment consisted of a between-subject design such that each score represented a separate animal. Injections of experimental drugs were given no more frequently than one every 7 days, and each rat received no more than three drug administrations. For a given experiment, each group was made up of separate subjects, but those subjects also appeared in some of the other interaction experiments.

2.5. Data analysis

Test data for individual rats were expressed as a percentage of the average of its own baseline pressing for the 3 days prior to injections, for both the punished and unpunished responding, and group means were then determined. No change from baseline pressing yielded 100% of baseline responding. An anxiolytic-like response would be reflected during the conflict component in a responding above baseline levels (i.e., more pressing or 'anti-conflict' effect) and an anxiogenic-like response would be reflected in a reduction below the baseline pressing.

For the dose–effect curve of allopregnanolone, a one-way analysis of variance (ANOVA) was performed. In the case of the allopregnanolone/drug interaction experiments, the scores for each dose were subjected to a two-factor ANOVA with drug as one factor and allopregnanolone or vehicle as the other. Individual means comparisons were made using the Dunnett's multiple comparison or the Newman-Keuls post-hoc tests. Significance is defined as $P < 0.05$.

3. Results

The effects of allopregnanolone on responding in the conflict test are shown in Fig. 1. Allopregnanolone produced a dose-dependent increase in the conflict (i.e., punished) component ($F(2,23) = 12.51$, $P < 0.05$). These effects were significant at the 8 mg/kg dose. A significant linear trend was also noted ($P < 0.0001$). A mild, but insignificant, stimulatory effect on lever pressing was observed in the unpunished component of the conflict test, similar to that seen with other anxiolytic compounds (Koob et al., 1986). Actual average baseline response rates were 379 lever presses/session in the unpunished component and 16.6 lever presses/session in the punished component of the conflict test. These baseline response rates are typical values for the Geller-Seifter conflict test modified for incremental shock.

Fig. 2 shows the interaction of allopregnanolone (8

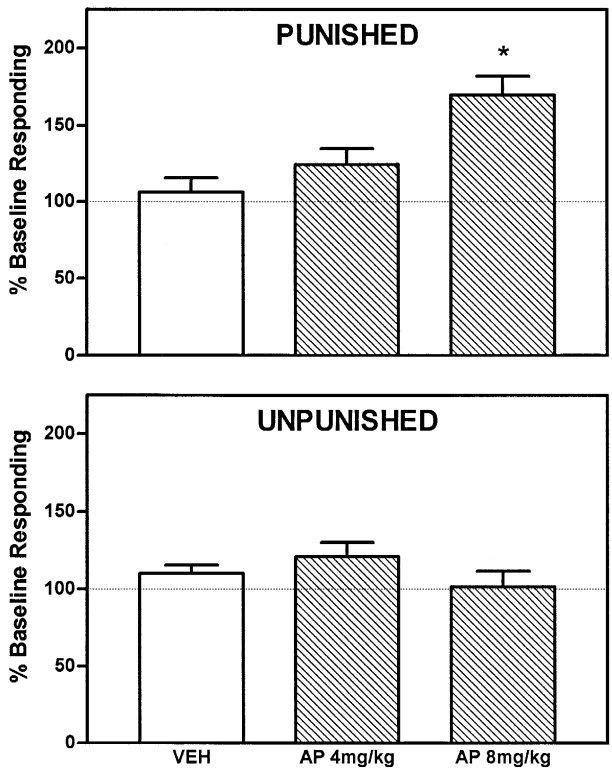

Fig. 1. Dose–effect curve of allopregnanolone on punished and unpunished responding in an operant conflict test. Results are expressed as percentage of baseline responding from previous 3 days (mean ± S.E.M.). $n = 6$ for vehicle (VEH), $n = 10$ for 4 mg/kg and $n = 10$ for 8 mg/kg doses of allopregnanolone (AP). * Significantly different from vehicle, $P < 0.05$. ANOVA followed by Newman-Keuls post-hoc test.

mg/kg) with the benzodiazepine receptor antagonist flumazenil. The doses of flumazenil were chosen on the basis of prior experiments in this laboratory as doses that, by themselves, do not alter unpunished responding in the conflict test (Britton et al., 1991). An ANOVA revealed a significant main effect for allopregnanolone ($F(4,48) = 5.41$, $P < 0.05$). However, flumazenil failed to attenuate significantly the increased rates of conflict responding produced by allopregnanolone. Also, no significant effects on responding were noted in the unpunished component of the conflict test ($P < 1$).

The effects of RO15-4513, a benzodiazepine receptor inverse agonist, on responding in the conflict test are shown in Fig. 3. Allopregnanolone (8 mg/kg) produced the expected significant increase in conflict responding ($F(3,20) = 7.79$, $P < 0.05$). RO15-4513 produced a dose-dependent attenuation of allopregnanolone's anti-conflict effects, reaching significance at 1.5 mg/kg. There were no significant changes in unpunished responding ($F(3,20) = 1.99$, n.s.). To determine whether RO15-4513 might produce its effects via an intrinsic, generalized rate-reducing action, the effects of RO15-4513 alone on responding in the conflict test were examined (Table 1). No effect on responding in the conflict component of the paradigm was noted. At these doses RO15-4513 produced a small, but significant, increase in responding in the random interval component at the 0.75 mg/kg dose.

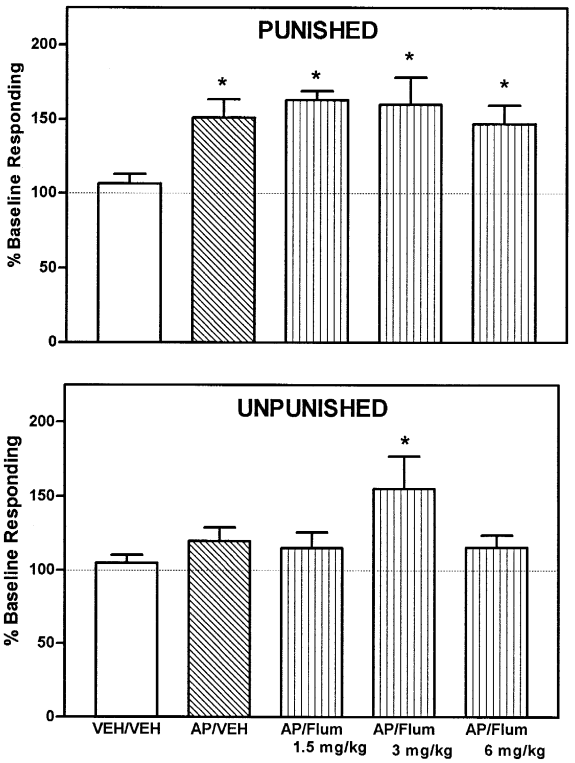

Fig. 2. Interaction between allopregnanolone and the benzodiazepine receptor antagonist flumazenil on punished and unpunished responding in the conflict test. $n = 16$ for vehicle (VEH), $n = 9$ for allopregnanolone (AP) and 1.5 mg/kg flumazenil (Flum), $n = 10$ for 3 and 6 mg/kg flumazenil. * Significantly different from vehicle, $P < 0.05$ ANOVA followed by Newman-Keuls test.

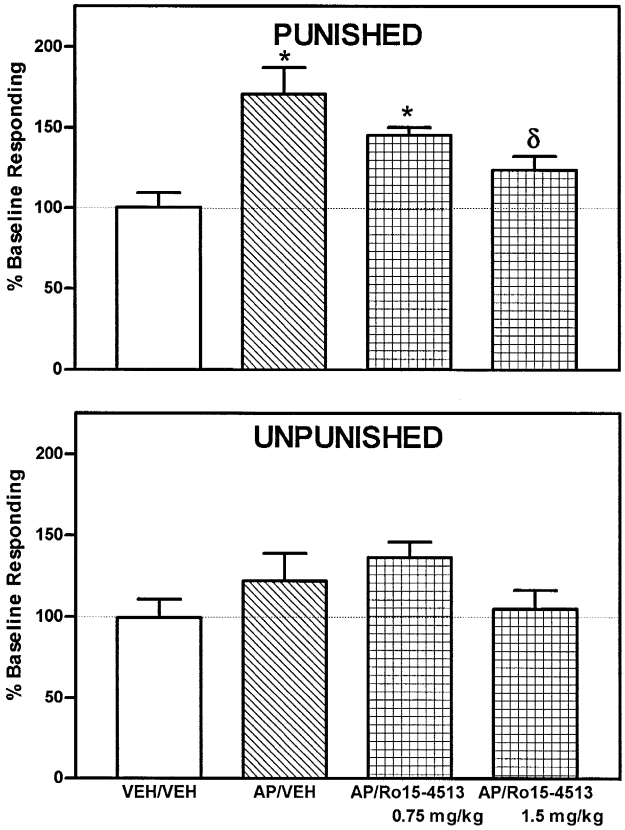

Fig. 3. Interaction between allopregnanolone and the benzodiazepine receptor inverse agonist RO15-4513 on punished and unpunished responding in the conflict test. $n = 5$ for vehicle (VEH) and allopregnanolone (AP), $n = 7$ for 0.75 mg/kg and $n = 10$ for 1.5 mk/kg doses of RO15-4513. * Significantly different from vehicle, $P < 0.05$. δ Significantly different from allopregnanolone, $P < 0.05$. Both ANOVAs followed by Newman-Keuls test.

Fig. 4 shows the effects of the chloride channel blocker isopropylcyclophosphate on responding in the conflict test. Isopropylcyclophosphate at doses lower than 20 μg/kg was previously reported to show no effect on its own either on the punished or unpunished component of the conflict test (Koob et al., 1989). In the present experiments, isopropylcyclophosphate produced a dose-dependent reduction of the increased responding produced by allopregnanolone in the conflict test, reaching significance at the 10 and 15 μg/kg dose ($F(4,20) = 3.60$, $P < 0.05$).

Table 1
Intrinsic effects of RO15-4513 on responding in the conflict test

Dose	% Baseline responding	
	Punished	Unpunished
Vehicle	98.9 ± 7.1 ($n = 7$)	75.6 ± 7.4 ($n = 7$)
0.75 mg/kg RO15-4513	99.0 ± 4.3 ($n = 8$)	97.3 ± 5.4 ($n = 8$)
1.5 mg/kg RO15-4513	97.6 ± 4.3 ($n = 7$)	67.8 ± 4.3 ($n = 7$)

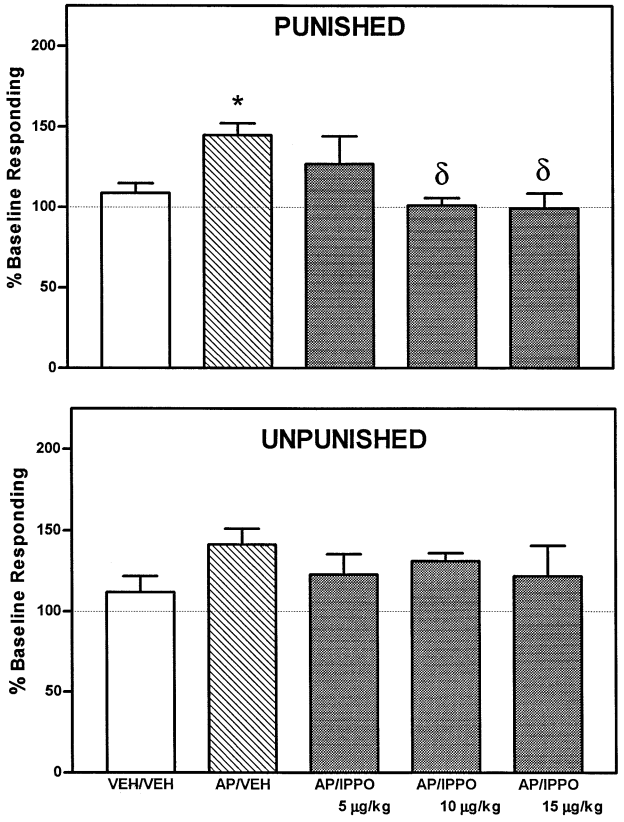

Fig. 4. Interaction between allopregnanolone and isopropylcyclophosphate (IPPO) on punished and unpunished responding. $n = 6$ for vehicle (VEH), $n = 5$ for allopregnanolone (AP), $n = 5$ for 5 μg/kg, $n = 10$ for 10 μg/kg and $n = 5$ for 15 μg/kg doses of isopropylcyclophosphate. * Significantly different from vehicle, $P < 0.05$. δ Significantly different from allopregnanolone, $P < 0.05$. ANOVAs followed by Newman-Keuls test.

In contrast, no effect on non-punished responding was noted at any of the isopropylcyclophosphate plus allopregnanolone doses tested.

4. Discussion

In the present study, allopregnanolone produced a robust anti-conflict action as measured by increased punished responding in the conflict test. At a dose of 8 mg/kg, rats significantly increased lever pressing during the conflict component of the test, while the unpunished (non-shocked) responses remained unaffected as in the vehicle-treated animals. These results show that allopregnanolone produces anxiolytic-like behavioral changes consistent with those observed in other animal models of anxiety. Allopregnanolone has been shown to possess hypnotic, anticonvulsant, and anxiolytic properties (Norberg et al., 1987; Belelli et al., 1989; Wieland et al., 1991; Bitran et al., 1993; Concas et al., 1996). For example, allopregnanolone administered to mice tested in the light/dark transition, open-field, and lick-suppression paradigms produced significant anxiolytic-like effects as compared to control mice (Wieland et al., 1991) as well as in non-incremental shock of the Geller-Seifter test and the elevated plus-maze test in rats (Wieland et al., 1995). Similar results have been obtained with the structurally related steroids, alphaxalone (Britton et al., 1991) and 3α,21-dihydroxy-5α-pregnan-20-one (Crawley et al., 1986; Wieland et al., 1991).

The mechanism of action underlying the anxiolytic-like behavioral effects of allopregnanolone was explored in the present study by examining the effects of different ligands of $GABA_A$ receptors on the anti-conflict effects of allopregnanolone. The benzodiazepine receptor antagonist, flumazenil, at doses of 3 and 6 mg/kg, failed to reverse the anti-conflict effect of allopregnanolone. In a previous study, these doses of flumazenil, when tested alone, did not alter responding on either component of the conflict test but did reverse the anti-conflict effects of the benzodiazepine, chlordiazepoxide (Britton et al., 1991). Similar findings have been reported by Wieland et al. (1991) who showed that pretreatment with flumazenil did not influence the anxiolytic-like effect of allopregnanolone whereas it reversed the anxiolytic-like effects of benzodiazepine in the light/dark transition, open-field, and lick-suppression paradigms. Thus the anxiolytic-like effects of allopregnanolone appear to be mediated at site(s) on $GABA_A$ receptors independent of the benzodiazepine site.

To explore further the possible mechanism of action for allopregnanolone, the benzodiazepine receptor partial inverse agonist, RO15-4513, was tested in combination with allopregnanolone. RO15-4513 has been shown to antagonize some of the behavioral effects of ethanol (Suzdak et al., 1986; Dar, 1995), including in vivo oral self-administration (Rassnick et al., 1993) and in vitro chloride uptake at $GABA_A$ receptors (Mehta and Shank, 1995). In the conflict test, RO15-4513 administered at doses of 0.75 and 1.5 mg/kg resulted in a dose-dependent reduction in the anti-conflict effect induced by allopregnanolone (8 mg/kg). When tested on its own in the present study at these low doses, RO15-4513 did not significantly affect the punished or unpunished component of the conflict test.

Benzodiazepine receptor agonists as well as barbiturates potentiate GABA's effects, while inverse benzodiazepine agonists decrease the efficiency of GABAergic synaptic transmission. Benzodiazepine receptor antagonists lack intrinsic activity, but bind to the benzodiazepine site of the $GABA_A$ receptors, thus preventing agonists or inverse agonists from binding. It has been reported that some 3α-hydroxy-ring A reduced steroids, including allopregnanolone, can modulate the $GABA_A$ receptors in a manner similar to barbiturates (Majewska et al., 1986). Allopregnanolone was demonstrated in vitro to act at distinct binding site(s) from those of barbiturates and benzodiazepine (Gee et al., 1988; Peters et al., 1988; Turner and Simmonds, 1989). In addition, it is likely that these 3α-hydroxy-ring A reduced steroids may have multiple sites of

action (Majewska et al., 1990; Prince and Simmonds, 1993). Recently, the existence in the brain of an additional benzodiazepine site on the $GABA_A$ receptors has been demonstrated. This site shows a relatively higher affinity for RO15-4513 than for flumazenil (Mehta and Shank, 1995). The behavioral evidence presented here indicates that RO15-4513 is capable of blocking the actions of allopregnanolone. The site for this action could be directly at the RO15-4513 site or could be through an allosteric interaction.

Another possible mechanism for the 'anxiolytic' effects of allopregnanolone may involve the picrotoxinin site of $GABA_A$ receptors. The picrotoxinin-site ligand isopropylcyclophosphate was previously reported to show no effect on its own either on the punished or unpunished component of the conflict test, at doses lower than 20 μg/kg (Koob et al., 1989). In the present studies, doses of 10 and 15 μg/kg of isopropylcyclophosphate were able to reverse the anti-conflict effect of allopregnanolone (8 mg/kg). This is consistent with other studies that showed a picrotoxinin-ligand induced reversal of the anti-conflict produced by benzodiazepine in the Geller-Seiter conflict paradigm (Billingsley and Kubena, 1978; Sepinwall and Cook, 1978). Since isopropylcyclophosphate has previously been demonstrated to reverse the anti-conflict effect of ethanol, the anxiolytic-like effects of allopregnanolone may be similar on some level to those of ethanol (Grant et al., 1996).

In summary, the present data provide additional evidence that allopregnanolone has potent anxiolytic-like effects, which are likely to be mediated at site(s) at the GABA/benzodiazepine receptor-chloride ionophore complexes independent from the benzodiazepine site and possibly at or nearby the picrotoxinin site. The possibility that neuroactive steroids can modulate $GABA_A$ receptors at sites independent of the benzodiazepine site not only suggest a means by which the organism modulates GABA tone but also provides a novel potential site(s) for therapeutic interventions.

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