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The Hyperphagic Effect of 3α-Hydroxylated Pregnane Steroids in Male Rats

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CHEN, S.-W., L. RODRIGUEZ, M. F. DAVIES AND G. H. LOEW. *The hyperphagic effect of 3α-hydroxylated pregnane steroids in male rats.* PHARMACOL BIOCHEM BEHAV **53**(4) 777–782, 1996.—Like benzodiazepines receptor (BDZR) ligands, 3α-hydroxylated, 5α, or 5β pregnane steroids are sedative, anticonvulsant, and anxiolytic. BDZR ligands also modulate the feeding response. Therefore, in this study we have investigated the effects of four 3α-hydroxylated pregnane steroids—Pregnanolone (3α-hydroxy-5β-pregnan-20-one), allopregnanolone (3α-hydroxy-5α-pregnan-20-one), alphaxalone (3α-hydroxy-5α-pregnan-11,20-dione), and 5β-pregnanediol (5β-pregnan-3α,20α-diol) on food intake. In non-food deprived male rats, all four steroids increased the consumption of a palatable diet. For pregnanolone (1–10 mg/kg), hyperphagia was found at lower doses than its anxiolytic effect (5–10 mg/kg) as determined using the elevated plus maze test. The presumed steroid antagonists, isopregnanolone (3β-hydroxy-5α-pregnan-20-one) (10 mg/kg) and pregnenolone sulfate (2 mg/kg), and the BDZ antagonist, Ro15-1788 (20 mg/kg), did not reverse the hyperphagic effect of pregnanolone. Picrotoxin, a $GABA_A$ receptor antagonist, dose dependently and at a subconvulsive dose (1.5 mg/kg), reversed the hyperphagic effect of pregnanolone and alphaxalone, but had no effect on allopregnanolone- and 5β-pregnanediol-induced hyperphagia. These results indicate that the hyperphagic effects of pregnanolone and alphaxalone are mediated by the $GABA_A$ receptor but not by direct interaction with BDZ receptors. However, allopregnanolone- and 5β-pregnanediol-induced hyperphagia may be mediated by other receptor systems. Because some 3α-hydroxylated pregnane steroids are endogenous progesterone metabolites, they may play an important role in appetite control.

Neuroactive steroids 5β-Pregnanediol Pregnanolone Allopregnanolone Alphaxalone
$GABA_A$ receptor Food intake

ENDOGENOUS progesterone metabolites such as 3α-hydroxylated pregnane steroids have long been known to have anesthetic and anticonvulsant effects (33). The continuing development of neurosteroids as clinical agents had led to the discoveries of the pattern of a functional-structure relationship and of the understanding of the structural requirements of binding to the progestin or mineralocorticoid receptors that are not relevant to their anesthetic or anticonvulsant effects (11). The 3α-hydroxylated pregnanes of both 5α and 5β configuration, were found to be highly effective anesthetics and anticonvulsants. The anesthetic and anticonvulsant activity were found to be similar between these 3α-hydroxylated pregnane steroids (2,11). However, the 3β-hydroxylated pregnane steroids are devoid of these actions (11,15), indicating a stereospecific effect.

Recently, like benzodiazepines (BDZs), the 3α-hydroxylated pregnane steroids have been shown to modulate the GABA/BDZ receptor Cl^- ionophore complex (4,16,22). Nanomolar concentrations of these steroids were found to inhibit the binding of [^{35}S]-t-butylbicyclophosphorothionate ([^{35}S]TBPS) to the sites near the Cl^- channel (16,22) and to enhance GABA-induced Cl^- influx in synaptoneurosomes and GABA-evoked currents in neurons (27). GABA receptor antagonists such as picrotoxin and bicuccline can block these effects (22); however, the BDZ antagonist and inverse agonist are ineffective (28). These progesterone metabolites can also allosterically modulate the binding of [^{3}H]flunitrazepam to BDZ receptors (14,17). In many instances, the 3α-hydroxylated pregnanes and BDZ ligands have similar behavioral effects (9,14). For example, 3α-hydroxy-5β-pregnan-20-one (pregnanolone) (5,15,34), 5α-pregnan-3α,21-diol-20-one, or tetrahydrodeoxycorticosterone (5α-THDOC) (9), 3α-hydroxy-5α-pregnan-20-one (allopregnanolone or 3α-DHP) (35), and 3α-hydroxy-5α-pregnan-11,20-dione (alphaxalone) (6) have been reported to be anxiolytic. In addition, both neurosteriods and BDZ receptor ligands are hypnotic and have potent anti-

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convulsant activity. These similarities suggest a close association between BDZ and neurosteroid receptors.

Modulation of food intake is one of the well known BDZ receptor-mediated functions (8). BDZ receptor agonists increase, and inverse agonists decrease food intake. Antagonists such as Ro15-1788 have no effect but can reverse the function of both agonists and inverse agonists (7,8). Even though many effects of neuroactive steroids have been elucidated and in general found to be similar to those of BDZ receptor ligands, the effect of neuroactive steroids on food intake has not been studied. Some observations have suggested such a modulation, for example, that animals recovering from steroid anesthesia eat eagerly (11), and pregnant women with high levels of endogenous progesterone metabolites have increased food ingestion throughout pregnancy (18). Therefore, it is possible that endogenous neuroactive steroids can induce food intake, and may play an important role in appetite control.

To investigate this possibility, the effect of four 3α-hydroxylated pregnane steroids—pregnanolone, allopregnanolone, alphaxalone, and 5β-pregnanediol—on food intake has been studied. Allopregnanolone, an endogenous neurosteroid present in high concentrations, has been reported to have a similar effect as pregnanolone in inhibiting ([^{35}S]TBPS) binding (14,15), enhancing [^{3}H]flunitrazepam to BDZ receptors (14), potentiating GABA-induced Cl^- influx (2,29), and directly stimulating Cl^- ion current at micromolar concentration (22). Alphaxalone is a synthetic neuroactive steroid with full anaesthetic agonist activity (11), although its ability to inhibit ([^{35}S]TBPS) binding is about 10 times less than pregnanolone or allopregnanolone (2). Recently, the pregnanediols have been reported as partial agonists in the naval neuroactive steroid site in GABA receptor (1), because of their lack of ability to directly stimulate Cl^- ion current (1) and their limited efficacy as modulators of [^{35}S]TBPS and [^{3}H]flunitrazepam (25). For this reason, we have included one of the pregnanediols-5β-pregnanediol (5β-pregnan-3α,20α-diol) in this study.

To help determine the site of action, the ability of specific antagonists for various sites of the $GABA_A$ receptor to block the observed hyperphagic effect of these neurosteroids was examined. The $GABA_A$ antagonist, picrotoxin, was used to elucidate the involvement of $GABA_A$ receptor sites (4). Ro15-1788, the BDZ receptor antagonist, has been shown to block the effects of BDZ on feeding (8,10) and was used to detect the role of the BDZ receptor site. Two presumed neurosteroid antagonists, pregnenolone sulfate and isopregnanolone (3β-hydroxy-5α-pregnan-20-one, also called 3β-allopregnanolone, or allopregnenolone), were also used to further evaluate the site of modulation of neurosteroids with respect to food intake.

METHOD

Pregnanolone, isopregnanolone, allopregnanolone, alphaxalone, 5β-pregnanediol, pregnenolone sulfate, and picrotoxin were purchased from Sigma Chemicals (St. Louis, MO). Ro15-1788 was received from Hoffmann-LaRoche (Nutley, NJ) as gifts. Picrotoxin was injected using saline as the vehicle. All other drugs were given in a 40% w/v solution of Encapsin® (β-cyclodextrin) (American Maize, Indianapolis, IN) in deionized water. The neuroactive steroids were given 30 min before the assessment of behavioral activity. All candidate antagonists were given 15 min after the administration of the neurosteroid.

Sprague-Dawley male rats (Bantin & Kingman, Fremont, CA, or Harlan, San Diego, CA) weighing from 300–400 g were used for both the feeding and anxiolysis studies. The procedures of the feeding experiment was similar to previously reported (7), as described below. The test diet for the feeding experiment was maintenance rat chow (Purina Mill, #5012) modified for palatability by adding 15% sucrose (Purina Mill, #5729-D). All experiments were started between 1100 h and noon. The day before the test day a sham experiment was conducted to familiarize the rats with the experimental handling techniques and test diet. Animals were transferred to individual test cages in a dark testing room, illuminated with a 40 watt red light bulb and allowed to acclimatize for 1 h before the administration of the test drug. Thirty minutes after administration of the neurosteroid a preweighed plastic dish containing about 40 g of test diet was placed inside the test cages. The duration of the test was 60 min, and during this interval only the test diet was available. At the termination of the test, the animals were returned to their home cage and the remaining food in the cup was weighed. The amount of test diet consumed was determined to the nearest 0.1 g, with correction for spillage.

A computer-controlled elevated plus-maze test system was adapted to study the anxiolytic and anxiogenic properties of pregnanolone. The apparatus consisted of two open arms (50 $\times$ 10 cm) and two enclosed arms (50 $\times$ 40 $\times$ 10 cm) made from dark Plexiglas and connected by a central platform (10 $\times$ 10 cm) mounted on a 50 cm high plastic base. The apparatus was equipped with 12 pairs of infrared photocell units and attached to an IBM computer. Thirty minutes following the drug or vehicle administration, the animal was placed in the center of the plus maze, facing a closed arm. The number of entries and the time spent in the open and closed arms and the center were recorded over a 5-min period.

The behavioral results were analyzed with one-way ANOVA using the Statview® program; the treatment effect was separated using the Dunnett t-test.

RESULTS

As shown in Fig. 1, pregnanolone dose dependently and at doses $\geq$1 mg/kg increased palatable food intake, $F(5, 59) = 11.985$, $p = 0.0001$, in nondeprived male rats. Pregnanolone also showed a significant anxiolytic effect in the elevated plus-maze test, $F(5, 31) = 6.136$, $p = 0.0005$, although at slightly higher doses ($\geq$5 mg/kg) (Fig. 2).

The BDZ antagonist, Ro15-1788 (20 mg/kg) did not reverse the effect of pregnanolone, $t(1, 48) = 0.48$, as shown in Fig. 3A. Picrotoxin dose dependently reversed the hyperphagic effect of pregnanolone, $F(7, 90) = 7.97$, $p = 0.0001$, completely antagonizing the hyperphagic effect of pregnanolone at 1.5 mg/kg (Fig. 3B). Two presumed steroid antagonists, 10 mg/kg of isopregnanolone, $t(1, 48) = 0.58$, and 2 mg/kg of pregnenolone sulfate, $t(3, 26) = 0.516$, were found to have no effect on the hyperphagic action induced by 10 mg/kg of pregnanolone, and by themselves did not affect food intake (Fig. 3C).

The effects of allopregnanolone, alphaxalone, and 5β-pregnanediol on food intake are shown in the Fig. 4. Allopregnanolone, at the 5 mg/kg dose, $F(3, 44) = 5.903$, $p = 0.0018$, alphaxalone, $F(5, 38) = 6.81$, $p = 0.0001$, and 5β-pregnanediol, $F(5, 43) = 5.12$, $p = 0.0009$, induced hyperphagia in nondeprived male rats. Picrotoxin (1.5 mg/kg) blocked the hyperphagic effect of alphaxalone, $t(3, 47) = 3.624$, $p <$

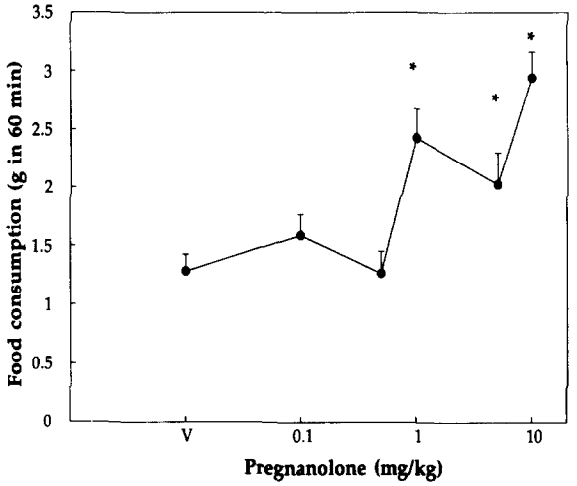

FIG. 1. Pregnanolone dose dependently increased palatable food intake in nondeprived male rats. Data are presented as mean ± SEM. V: vehicle-treated group. *Significantly different from vehicle-treated group.

0.05, but had no effect on either allopregnanolone, $t(3, 47) = 0.374$, or 5β-pregnanediol, $t(3, 53) = 0.44$ (Table 1).

DISCUSSION

The results of this study clearly demonstrated that 3α-hydroxylated neuroactive steroids increased food intake in nondeprived male rats. All four neuroactive steroids tested increased food intake significantly. Surprisingly, the putative partial agonist, 5β-pregnanediol, increased food intake at the lowest dose, 0.5 mg/kg, with no further increase observed up to 10 mg/kg. Pregnanolone and alphaxalone produced a

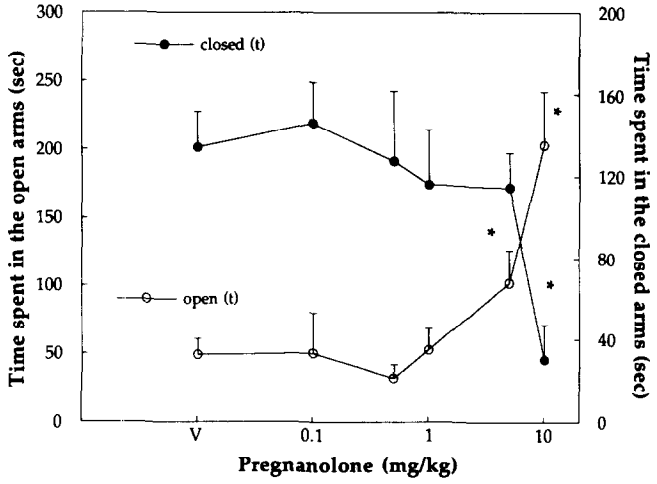

FIG. 2. The anxiolytic effect of pregnanolone as tested in the plus-maze apparatus. Pregnanolone increased time spent on the open arms (open circles) and decreased the time spent on the closed arms (closed circle). V: vehicle-treated group. *Significantly different from vehicle-treated group.

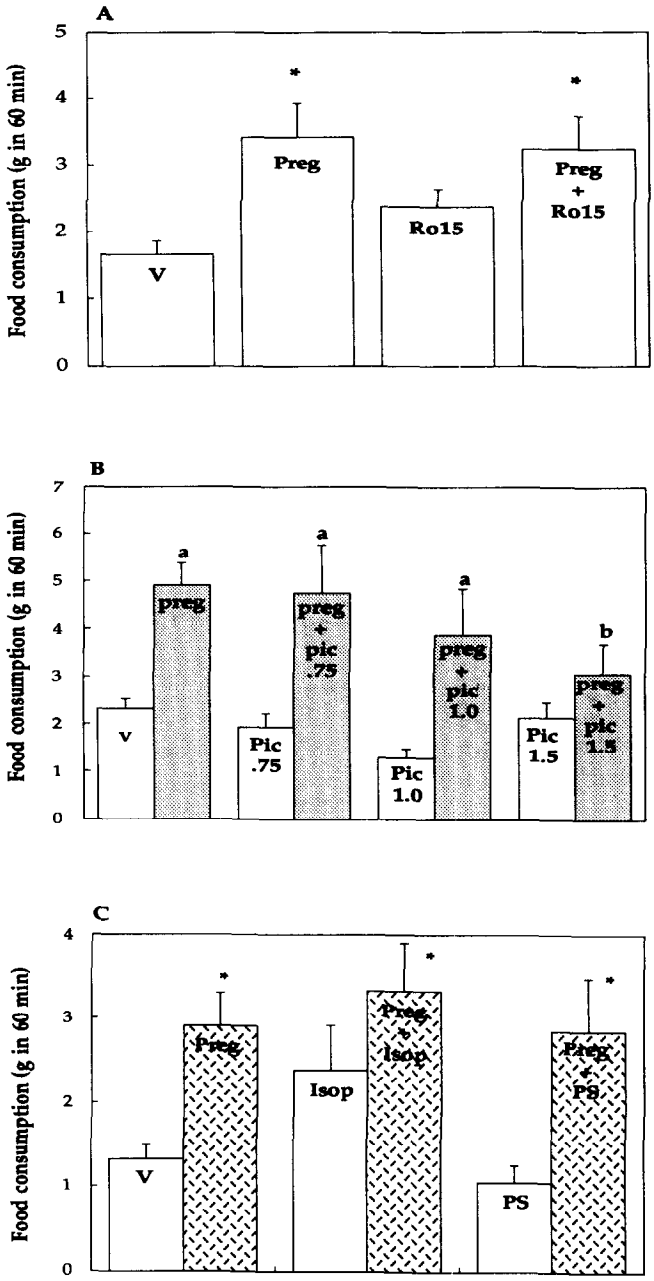

FIG. 3. Effects of candidate antagonists on pregnanolone (10 mg/kg) induced hyperphagia. (A) The benzodiazepine antagonist Ro15-1788 did not block the hyperphagic effect of pregnanolone. Ro15-1788 (Ro15) (20 mg/kg) was given 15 min after the administration of pregnanolone and had no effect on pregnanolone (Preg)-induced hyperphagia. (B) Subconvulsant doses of the $GABA_A$ antagonist, picrotoxin, dose dependently reversed the hyperphagic effect of pregnanolone. Picrotoxin 0.75 mg/kg (pic .75), 1 mg/kg (pic 1.0), and 1.5 mg/kg (pic 1.5) did not themselves affect food intake, but 1.5 mg/kg picrotoxin significantly blocked the hyperphagic effect induced by pregnanolone. (C) Isopregnanolone and pregnenolone sulfate did not reverse pregnanolone-induced hyperphagia. Isopregnanolone (Isop) (10 mg/kg) and pregnenolone sulfate (PS) (2 mg/kg) did not have an effect on food intake, and they did not affect pregnanolone-induced hyperphagia. V: Vehicle-treated group. [a]Significantly different from the vehicle-treated group. [b]Different from the pregnanolone-treated group.

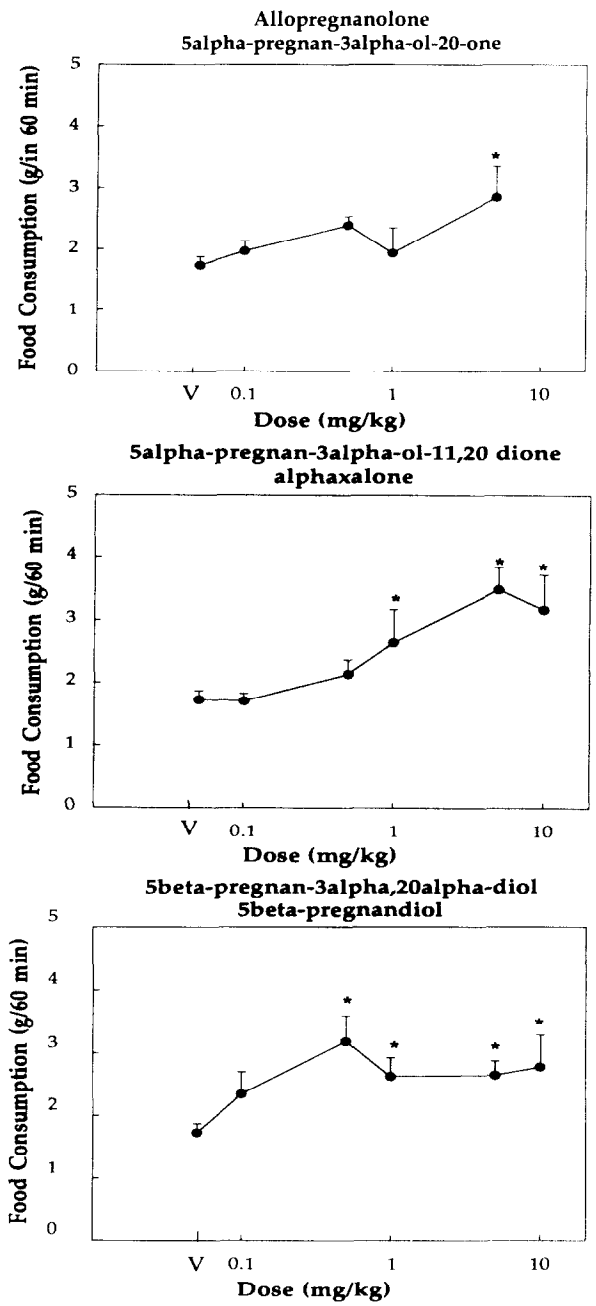

FIG. 4. Effects of allopregnanolone, alphaxalone, and 5β-pregnanediol on food intake in nondeprived male rats. V: Vehicle-treated group. *Significantly different from the vehicle-treated group.

significant dose-dependent hyperphagic effect, beginning at 1 mg/kg and allopregnanlone produced significant enhancement at 5 mg/kg.

The effective anxiolytic doses of 5–10 mg/kg pregnanolone found in this study are similar to those found for other neuroactive steroids using the same route of administration. For example: the effective anxiolytic dose reported for tetrahydroxycorticosterone in mice in lick suppression conflict test was 5–15 mg/kg (9) and alphaxolone required 6–8 mg/kg in the plus-maze test (6). The results of this study indicate that the hyperphagic effect is more robust than the anxiolytic effect for pregnanolone. A similar conclusion can be reached in the case of alphaxalone when comparing the effective doses of hyperphagic activity found here with the reported 6–8 mg/kg required to produce an anxiolytic effect in the plus-maze test (6). Therefore, it is possible that in general the hyperphagic effect of 3α-hydroxylated pregnane steroids is more profound than their anxiolytic effect.

Allopregnanolone and pregnanolone are 5α- and 5β isomers, respectively, of the same neurosteroid. Interestingly, the stereospecific preference for these isomers is different in different biochemical and behavioral end points. For example, allopregnanolone has been showed to be more effective in inhibiting [^{35}S]TBPS binding, and in enhancing [^{3}H]flunitrazepam affinity for BDZ receptors than pregnanolone (14,15). In two behavioral end points previously reported, induction of anesthesia (2) and anticonvulsant activity (3), the efficacies of these two isomers was similar. By contrast, and in the opposite direction at their effect on receptor binding, the 5β-isomer (pregnanolone) has been shown to possess more efficacious anxiolytic property (34) than 5α-isomer (allopregnanolone). We find the same order of potency for the two isomers in the hyperphagic activity. A low dose of pregnanolone (1 mg/kg) increases food intake, whereas allopregnanolone requires at least 5 mg/kg dose to exert an effect. Therefore, it seems that the hyperphagic activity of neuroactive steroids do not act in a way parallel to their modulation of binding affinities.

This conclusion is reenforced by the behavioral results obtained for alphaxalone. In general, the inhibitory potencies of neuroactive steroids in the [^{35}S]TBPS assay correlates well with their potencies for enhancing [^{3}H]flunitrazepam binding in rat cortical membrane (17) and with the in vitro activity in GABA-evoked currents. Because alphaxalone was reported to be about 10 times less effective compared to pregnanolone or allopregnanolone (2) in the binding assays and in its ability to potentiate Cl^- ion channel (34), we selected alphaxalone to compare its effect on feeding with pregnanolone and allopregnanolone. We have found that the hyperphagic function of alphaxalone is similar to pregnanolone and superior to allopregnanolone. This result reenforces our findings for allopregnanolone and pregnanolone that the hyperphagic activity of neuroactive steroids is not correlated with either in vitro activity assays or binding assays.

There is some evidence from in vitro studies that steroids

TABLE 1

EFFECT OF PICROTOXIN ON ALLOPREGNANOLONE, ALPHAXALONE, AND 5β-PREGNANEDIOL INDUCED HYPERPHAGIA

Treatment	Food Consumption (g in 60 min)
Vehicle + Saline	1.78 ± 0.420
Vehicle + 1.5 mg/kg picrotoxin	1.66 ± 0.225
Allopregnanolone (5 mg/kg) + Saline	2.86* ± 0.420
+ 1.5 mg/kg picrotoxin	2.71* ± 0.225
Alphaxalone (5 mg/kg) + Saline	3.48* ± 0.360
+ 1.5 mg/kg picrotoxin	2.00† ± 0.285
5β-pregnanediol (5 mg/kg) + Saline	2.50* ± 0.154
+ 1.5 mg/kg picrotoxin	2.37 ± 0.309

*Significantly different from vehicle + saline-treated group.
†Picrotoxin reversed the hyperphagic effect.

can exert both positive and negative effects on $GABA_A$ receptor function. Anxiolytic BDZs, sedative barbiturates, and 3α-hydroxylated steroids enhanced GABA-mediated synaptic inhibition and GABA-evoked Cl^- conductance (12,22), whereas pregnenolone sulfate (23,24) and the 3β-hydroxylated steroids (32) suppressed $GABA_A$ receptor-mediated events. At micromolar concentrations, pregnenolone sulfate antagonized the responses to GABA, by reducing the frequency of Cl^- channel opening (24). Pregnenolone sulfate also inhibited the binding of [^{35}S]TBPS and steroid-enhanced binding of [^{3}H]flunitrazepam (21). Other 3β-hydroxy steroids such as isopregnanolone, however, have been shown to have little effect on the inhibition of binding to [^{35}S]TBPS (15), to have no in vitro or in vivo activity (4,28), but to reverse the potentiating effect of allopregnanolone on [^{3}H]flunitrazepam binding (32). However, the in vivo effect of these presumed steroid antagonists has not been previously tested. The results of this study do not show antagonism either by isopregnanolone or pregnenolone sulfate to the hyperphagic effect of pregnanolone. One possible reason for this inconsistency of these in vivo results with the in vitro antagonism is the evidence that all the progesterone metabolites are converted to allopregnanolone in the brain (5,26). If these in vitro antagonists, indeed, are metabolized to 3α-hydroxylated steroids in vivo, the enzyme, 3α-hydroxysteroid oxidoreductase should be the key factor.

Because of the many functional similarities between BDZ receptor ligands and neurosteroids, we have assessed the ability of the BDZ receptor antagonist, Ro15-1788, to antagonize pregnanolone induced hyperphagia. Ro15-1788, at the dose used in this study (20 mg/kg), does not block the hyperphagic activity of pregnanolone, although this BDZ antagonist has been shown to reverse or reduce the effect of neuroactive steroid in defeat-induced analgesic effect (19). In addition, Ro15-1788 has been shown to be very effective in reversing both the anorexic and hyperphagic effects of BDZ receptor ligands (8,10). Previous studies demonstrated that 3α-hydroxylated steroids modulate the $GABA_A/Cl^-$ ion channel through a novel binding site separate from the BDZ receptor site (14). Further support for the observation from this study is the inability Ro15-1788 to block the hyperphagic effect of pregnanolone.

Although the interaction between $GABA_A$ receptor and neuroactive steroids have been repeatedly demonstrated in vitro, the effects of $GABA_A$ antagonists on neuroactive steroid function are not consistently observed in vivo. The anxiolytic effect of alphaxalone in the plus-maze test was not blocked by Ro15-1788, bicuccline, or picrotoxin, although was partially blocked by TBPS (6). However, another study by Bitran et al. (4) showed that ICV administration of pregnanolone produced an anxiolytic effect that was blocked by picrotoxin. Picrotoxin has also been shown to block the effect of pregnanolone on enhancement of [^{3}H]muscimol binding (20). This study, showed that picrotoxin dose dependently reversed the hyperphagic effect of pregnanolone and alphaxalone, indicating that it is a $GABA_A$ receptor-mediated function. Therefore, like the anesthetic (16), anxiolytic (4), and anticonvulsant (3) activities, the hyperphagic effect of pregnanolone and alphaxalone appeared to be mediated by $GABA_A$ receptors.

Interestingly, we have discovered that among the four hyperphagic neuroactive steroids tested, picrotoxin was unable to reverse the effect of allopregnanolone and 5β-pregnanediol, even though it clearly blocked the effect of pregnanolone and alphaxalone. The mechanism of allopregnanolone- and 5β-pregnanediol-induced hyperphagia is unclear. There is, however, emerging evidence that neuroactive steroids interact in other ligand-gated ion channels (29) and voltage-gated Ca^{2+} channels (13) and other receptor systems, such as glycine, NMDA receptor (31), and G-protein-coupled receptors (30). Neuroactive steroids differing in the substitute at the 5-pregnane position have been shown to display varied response in strychnine-sensitive glycine receptors (31). Therefore, one has to be very careful when investigated the functional activity of neuroactive steroids. Structurally similar analogs such as pregnanolone and allopregnanolone with similar functional profiles seem to have different mechanism of action.

In summary, this study is the first to demonstrate clearly that certain endogenous progesterone metabolites, 3α-hydroxylated pregnane steroids, are effective hyperphagic agents. In addition, these hyperphagic effects are found at lower doses than their anxiolytic effect. Furthermore, the hyperphagic effects of pregnanolone and alphaxalone are mediated by the $GABA_A$ receptor because the $GABA_A$ antagonist, picrotoxin, reversed these effects, but at a site distinct from the BDZ receptors. However, some other mechanisms cannot be ruled out, because the hyperphagic function of these steroids do not correlate well with the documented in vitro efficacies on $GABA_A$ receptor site. Finally, because the level of progesterone is high in the last trimester of gestation in woman, its natural metabolites, the 3α-hydroxylated neurosteroids may play an important role in appetite control. These results may also encourage the development of 3α-hydroxylated pregnane steroids as either appetite enhancing or suppressing agents.

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