Psychopharmacology (1996) 126 : 173–178

ORIGINAL INVESTIGATION

Ezio Carboni · Scott Wieland · Nancy C. Lan
Kelvin W. Gee

Anxiolytic properties of endogenously occurring pregnanediols in two rodent models of anxiety

Received: 16 December 1995/Final version: 4 January 1996

Abstract Certain endogenously occurring 3α-hydroxylated, 5-reduced pregnane steroids act at a specific site on the $GABA_A$ receptor complex (GRC) to modulate the effects of GABA at its receptor. Modulators that potentiate GABA at the $GABA_A$ receptor often possess anxiolytic properties. The anxiolytic potential of four 5-reduced, 3α, 20-pregnanediols, differing only in the stereochemical orientation of the steroid A-ring and the 20-hydroxyl group, were tested in the Vogel test following intracerebroventricular (ICV) administration. The effects of these pregnanediols were compared to those of their 20-ketone analogues, 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) and 3α-hydroxy-5β-pregnan-20-one (3α,5β-P). All four pregnanediols tested significantly enhanced punished drinking at doses ranging from 10 to 60 μg. The rank order of potency based on the minimum effective dose (MED) observed was 5α-pregnan-3α,20α-diol = 5β-pregnan-3α,20α-diol > 5β-pregnan-3α,20β-diol > 5α-pregnan-3α,20β-diol. 3α,5β-P and 3α,5α-P enhanced punished responding when administered at 2.5 and 5 μg, respectively. 3β,5α-P which is inactive at the GRC was also inactive (up to 100 μg) in the Vogel test. The benzodiazepine control diazepam was efficacious when administered at 2.5 μg. 5α-Pregnan-3α,20α-diol was further tested in the mouse elevated plus-maze model following systemic administration where it was found to be active in a dose range of 10–40 mg/kg IP. These results raise the possibility that in addition to 3α,5α-P and 3α,5β-P, some of their endogenously occurring pregnanediol metabolites may also influence physiological processes related to anxiety via the GRC.

E. Carboni · K.W. Gee (✉)
Department of Pharmacology, College of Medicine,
University of California Irvine, Irvine, CA 92717, USA

S. Wieland · N.C. Lan
Department of Pharmacology, CoCensys Inc.,
213 Technology Drive, Irvine, CA 92718, USA

Key words γ-Aminobutyric acid · $GABA_A$ receptor complex · 3α-Hydroxy-5α-pregnan-20-one · 3α-Hydroxy-5β-pregnan-20-one · 5α-Pregnan-3α,20α-diol · Benzodiazepines · Anxiolytic · Neuroactive steroids · Neurosteroids · Vogel test · Elevated plus-maze · Progesterone metabolites · Benzodiazepine receptor

Introduction

Anxiolytic activity is found among the diverse classes of drugs that act on the γ-aminobutyric $acid_A$ ($GABA_A$)-receptor chloride channel complex (GRC) to potentiate the effects of GABA on chloride channel conductance. It is now known that certain neurosteroids act specifically at the GRC to produce a variety of pharmacological effects, some of which appear to overlap those of the benzodiazepines (BZs), barbiturates, and certain GABA-mimetics. Sufficient evidence has now accumulated to suggest that these neuroactive steroids may be representatives of a novel class of ligands with high specificity for the GRC and the word "epalons" (i.e., an psuedoacronym for epiallopregnanolone) has been proposed to define this class (for a review, see Gee et al. 1995). Thus based on functional criteria, epalons, including both naturally and synthetically derived steroids, act at a unique site(s) distinct from those for BZs and barbiturates, to modulate allosterically the effects of GABA at the GRC (Lan et al. 1990; Puia et al. 1990). These neuroactive steroids allosterically inhibit [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) binding, increase binding of the $GABA_A$ agonist [^{3}H]muscimol to the GABA recognition site and enhance the affinity of [^{3}H]flunitrazepan binding to the BZ site in brain membrane homogenates (Majewska et al. 1986; Gee et al. 1987, 1988; Harrison et al. 1987; Peters et al. 1988).

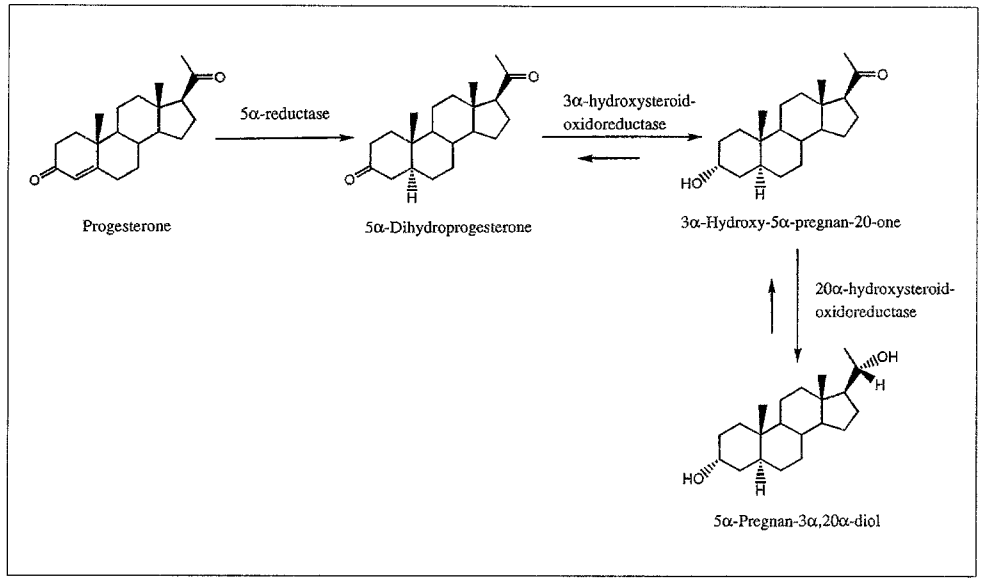

Fig. 1 Metabolic pathway for the enzymatic conversion of progesterone to 3α-hydroxy-5α-pregnan-20-one and 5α-pregnan-3α,20α-diol. The formation of the 5β- and 20β-reduced isomers result from the action of 5β-reductase and 20β-hydroxysteroid-oxidoreductase, respectively

GRC-active 3α-hydroxylated, 5α(β)-pregnan-20-one steroids can be formed in brain via the action of specific reductase enzymes on progesterone (Corpechot et al. 1993). These neuroactive steroids can be further metabolized by 20-hydroxysteroid dehydrogenase to 5β-pregnan-3α,20α-diol (5β,3α,20α-diol) 5α-pregnan-3α,20α-diol (5α,3α,20α-diol), 5β-pregnan-3α,20β-diol (5β,3α,20β-diol) and 5α-pregnan-3α,20β-diol (5α,3α,20β-diol). As an example, the pathway for the endogenous formation of 5α,3α,20α-diol from progesterone is illustrated in Fig. 1. Like their GRC-active precursors, all four of the pregnanediols have the capability of allosterically modulating [^{35}S]TBPS and [^{3}H]flunitrazepam binding to brain homogenates and potentiate ^{36}Cl-uptake into brain synaptoneurosomes in manners consistent with GABA-mimetic pharmacology (Belelli and Gee 1989; McCauley et al. 1995). Furthermore, two of these pregnanediols, 5α,3α,20α-diol and 5β,3α,20β-diol appear to have limited efficacy in these in vitro assays. Coupled with evidence that the precursors to the pregnanediols, progesterone, 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) and 3α-hydroxy-5β-pregnan-20-one (3α,5β-P), are anxiolytic in animal models (Rodriguez-Sierra et al. 1984; Bitran et al. 1991, 1995; Wieland et al. 1991), it is likely that these pregnanediols may also have anxiolytic properties. Furthermore, the observation that some of these reduced steroids are also formed endogenously has raised the question of whether they play a role in the physiological regulation of anxiety via the GRC (Arafat et al. 1988).

In the present study, the anxiolytic properties of four pregnanediols were compared to their metabolic precursors 3α,5β-P and 3α,5α-P in the rat Vogel test (Vogel et al. 1971). 5α,3α,20α-Diol was further tested in the mouse elevated plus-maze (Pellow et al. 1985) model of anxiety.

Materials and methods

Male Sprague-Dawley rats (~ 300 g) were individually housed at 23 ± 1°C under a normal 12-h light-dark cycle with free access to food and water except as indicated for the Vogel test.

Intracerebroventricular cannulae implantation

Guide cannulae (C313G, Plastic One, Roanoke, Va.) were aseptically implanted in the lateral ventricle (coordinates from Paxinos and Watson (1986): A = 0.9; L = 1.3; V = 3.3 mm from dura), under ketamine (Western Medical Supply, Arcadia, Calif.; 80 mg/kg IP) and xylazine (Western Medical Supply; 10 mg/kg IP) anesthesia. Rats were allowed to recover for 1 week followed by training for the Vogel test (Vogel et al. 1971). The intracerebroventricular (ICV) route of administration was chosen initially to limit the influence of absorption barriers and metabolic degradation on drug response. All steroids and BZs used in the present study were obtained from CoCensys (Irvine, Calif., USA) and Sigma (St Louis, Mo., USA), respectively.

Rat Vogel test

A slightly modified version of the Vogel procedure was used. Briefly, the rats were trained for 3 days and the test was performed on day 4 only. Rats had no access to water at 1600 hours on day 1 and limited access to water (1300–1600 hours) on days 2 and 3. On the morning of day 2, rats were exposed to the test cage (Anxiomonitor, Omnitech Electronics, Columbus, Ohio, USA) and were allowed to drink freely for 10 min (i.e., the pre-test). The number of licks and time spent in the cage before drinking (i.e., delay) were recorded. Rats that did not drink within 10 min were not included in this study. Rats that drank in the pre-test were assigned randomly to a vehicle control or drug treatment groups consisting of at least eight rats per group. The average number of licks and the average delay were not significantly (by analysis of variance) different among the groups. Rats were tested on day 4. Drugs were dissolved by sonication in 60% hydroxypropyl-γ-cyclodextran (RBI, Natick, Mass.) in normal saline at a concentration of 4 mg/ml and then diluted with the same vehicle according to the dose to be administered. The

drug vehicle or test drug was injected ICV. Rats received 10 µl injections over 6 min while in their home cage using an electric pump driven 25 µl Hamilton syringe (Stoelting, Wood Dale, Ill., USA). Two minutes later, rats were exposed to the test cage where they received a 0.8 mA shock for every 25 licks. Rats were left in the cage for 10 min after the first lick. The number of licks and the delay were recorded. Separate groups of rats were tested for each drug dose or vehicle. Data were expressed as the number of licks during the 10-min period following the first lick. Data analysis utilized the *t*-test for comparisons between a drug dose and its own vehicle control. Comparisons between several drug doses and a common vehicle control utilized ANOVA followed by Dunnett's *t*-test for post-hoc comparisons. The minimum effective dose (MED) was defined as the lowest dose above a no-effect dose showing a statistically (*t*-test) significant difference from vehicle controls.

Mouse elevated plus-maze

Male NIH Swiss-Webster mice weighing 20–30 g were used in this model of anxiety. Animals were housed in groups of four at 23° ± 1°C under a 12-h light-dark cycle with free access to food and water. Drugs were micronized and suspended in a solution containing 0.35% hydroxypropylmethylcellulose (Sigma), 4.0% Tween-80 (Sigma), and normal saline. The method used was described previously (Pellow et al. 1985). The apparatus included two open arms perpendicular to two enclosed arms elevated 50 cm from the floor. Each arm was 50 cm long and the walls of the enclosed arms were 40 cm tall. The maze was made completely of black Plexiglas. Incandescent 200 W light bulbs were positioned above each of the open arms to produce a strong contrast between the open arms and the enclosed arms.

Ten minutes after an IP injection, the mice were placed in the center of the plus-maze facing an open arm. During the 5-min test period, the number of entries onto the open arms and the enclosed arms, and the time spent in the open arms and enclosed arms were recorded. All four paws had to be within an arm for the dependent variable to be measured. Therefore, the time spent in the center of the maze is not counted, so the total spent in the open arms and the enclosed arms may not equal 5 min. All observations were made by the same observer who was blinded regarding treatment conditions. Dunnett's *t*-test was used for comparisons between drug and vehicle control groups.

Chemical structure

Figure 2 illustrates the structural relationships between the GRC-active steroids used in this study. All the neurosteroids tested belong to two groups with different chemical composition. They either have 20-ketone or 20-hydroxyl substituents. The remaining differences are stereochemical, in that the orientation of the 5-H, 3-hydroxyl and 20-hydroxyl groups is either α or β. The hydrogen group in position 5 determines the orientation of the steroid A-ring relative to the B ring.

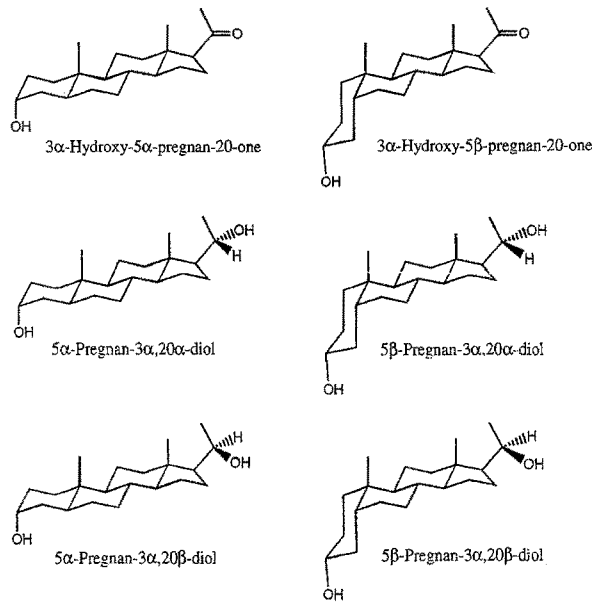

Fig. 2 Chemical structures of the various GRC-active neurosteroids tested

Results

None of the drugs at the doses tested had any significant effect on unpunished drinking (data not shown). 3α,5α-P increased punished drinking at both 5 and 10 µg but had no effect at a dose of 2.5 µg (Fig. 3A). The 5β isomer 3α,5β-P was more potent as it was able significantly to increase punished drinking at a dose of 2.5 µg (Fig. 3B). 3β,5α-P, a steroid that is inactive at the GRC, did not show any effect in the Vogel test when injected at doses up to 100 µg (Fig. 3C). For the positive control diazepam, the dose of 2.5 µg ICV produced a significant increases in punished drinking (Fig. 3D). In contrast, 1 µg diazepam was inactive, thus suggesting that its MED under the conditions used was between 1 and 2.5 µg. The effects (i.e., percentage of vehicle control licking) reached at the highest doses of 3α,5α-P and 3α,5β-P tested were greater than that for diazepam.

The effects of the four pregnanediols in the Vogel test are shown in Fig. 4. Based on apparent MEDs, two of four pregnanediols were approximately equipotent in this test. 5α,3α,20α-Diol and 5β,3α,20α-diol significantly enhanced punished drinking at a dose of 10 µg, whereas 5β,3α,20β-diol was active at 20 µg (Figs. 4A, 4B and 4C, respectively). In contrast, 5α,3α,20β-diol (Fig. 4D) enhanced punished drinking only at the highest dose tested of 60 µg. The most potent (i.e., based on modulation [^{35}S]TBPS binding) pregnanediol, 5α,3α,20α-diol, was chosen for evaluation in the mouse elevated plus maze. The positive control, chlordiazepoxide, was active at 10 mg/kg, 5α,3α,20α-Diol was also systemically active at doses as low as 5 mg/kg IP (Table 1).

Discussion

It is now well established that rat brain and pituitary can metabolize progesterone to 5α-reduced products (Karavolas et al. 1984). Δ^4-5α-Reductase catalyzes the reduction of progesterone to 5α-preganane-3,20-dione (5α-hydroxy-progesterone) which in turn can be

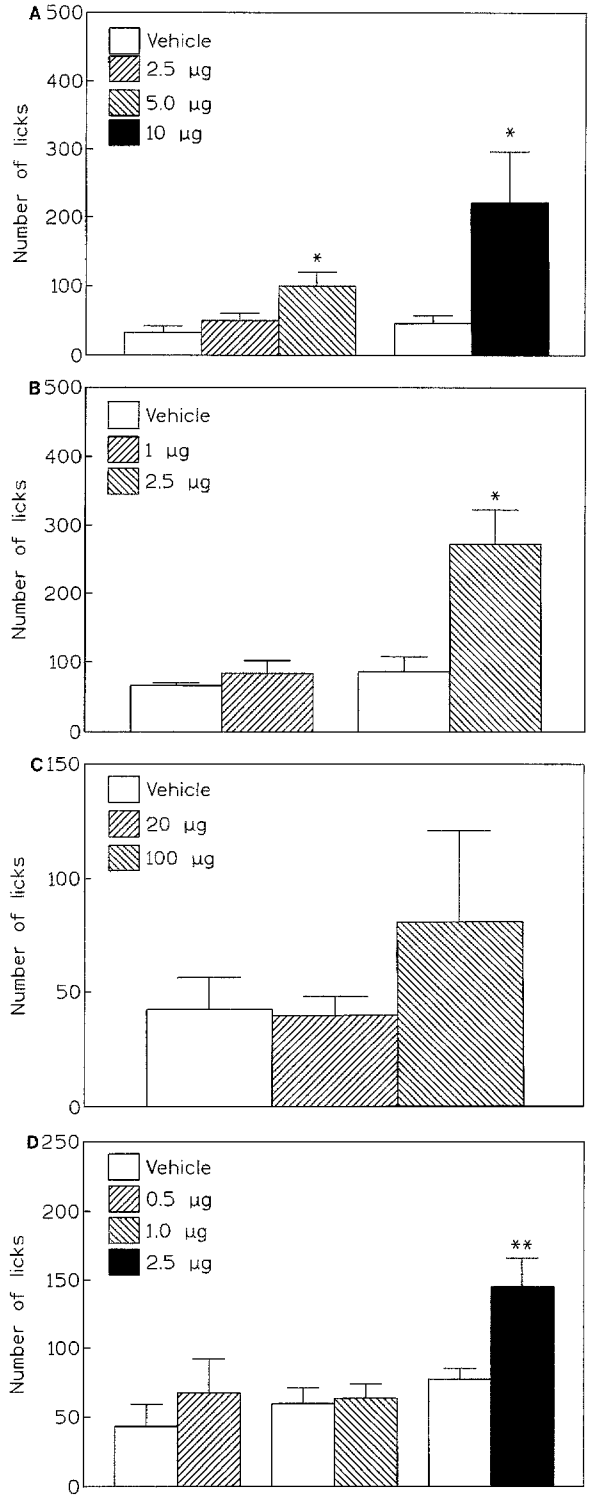

Fig. 3A–D The effects of 3α-hydroxy-5α-pregnan-20-one **A**, 3α-hydroxy-5β-pregnan-20-one **B**, 3β-hydroxy-5α-pregnan-20-one **C**, and diazepam **D** in the Vogel test. *Columns* represent punished drinking expressed as the number of licks in the 10-min period following the first lick. Rats received a shock of 0.8 mA every 25 licks. Each column in the mean ± SE of the results obtained from at least eight rats. $^{*}P < 0.05$ and $^{**}P < 0.01$, by *t*-test or Dunnett's *t*-test where appropriate

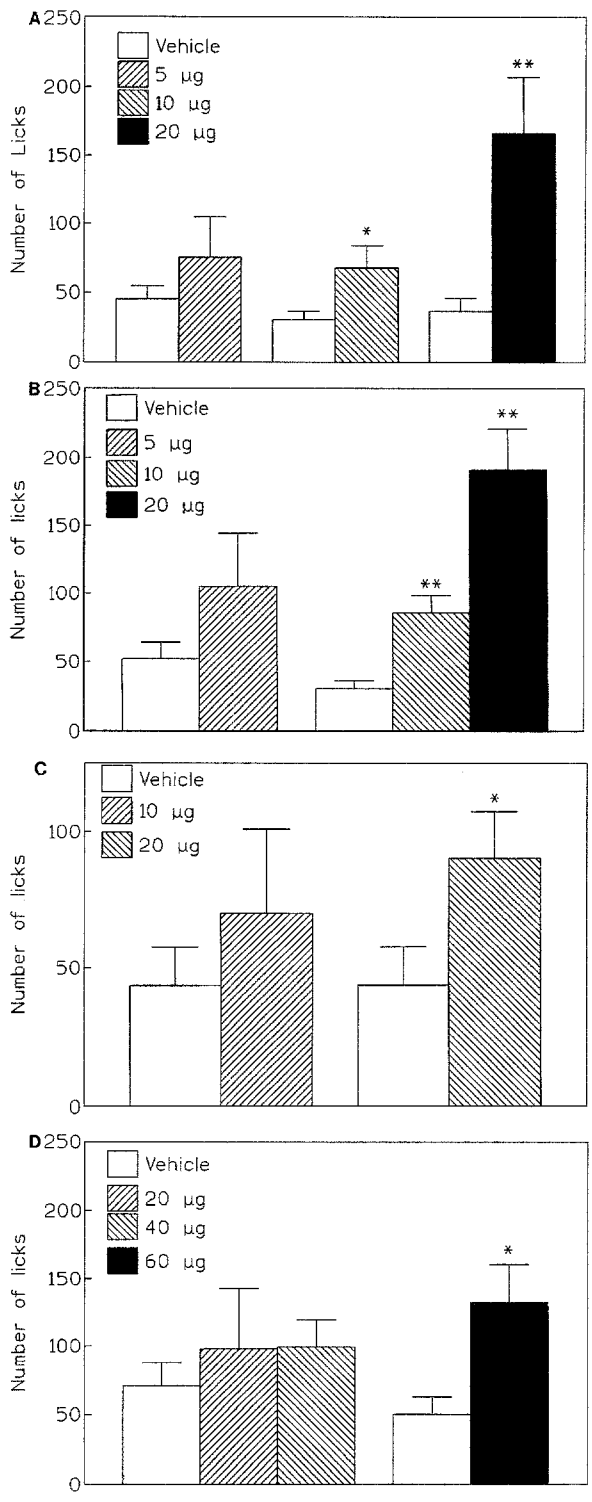

Fig. 4A–D The effects of 5α-pregnan-3α,20α-diol **A**, 5β-pregnan-3α,20α-diol **B**, 5β-pregnan-3α,20β-diol **C**, 5α-pregnan-3α,20β-diol **D** in the Vogel test. *Columns* represent punished drinking expressed as the number of licks in the 10-min period following the first lick. Rats received a shock of 0.8 mA every 25 licks. Each column in the mean ± SE of the results obtained from at least eight rats. $^{*}P < 0.05$ and $^{**}P < 0.01$, by *t*-test or Dunnett's *t*-test where appropriate

Table 1 The effect of 5α-pregnan-3α,20α-diol (5α,3α,20α-diol) and chlordiazepoxide on the percentage of entries into the open-arm and the percentage of time spent in the open-arm expressed as the mean ± SE in the mouse elevated plus-maze. Number mice tested at each dose was 10

Drug	Dose (mg/kg, IP)	% Open entries	% Time
Vehicle	0	20.9 ± 5.1	12.4 ± 4.3
5α,3α,20α-diol	5	48.4 ± 6.8**	36.6 ± 8.6*
	10	40.5 ± 5.6*	33.2 ± 7.2
Chlordiazepoxide	10	64.3 ± 5.1**	61.8 ± 7.1**

Values for 5α,3α,20α-diol and chlordiazepoxide are significantly different from vehicle at $^{*}P < 0.05$ and $^{**}P < 0.01$ level of significance or greater by Dunnett's *t*-test

further reduced by a 3α-hydroxysteroid-dehydrogenase to 3α,5α-P. Via an alternative route, progesterone can be converted by a 20α-hydroxysteroid-dehydrogenase to 20α-hydroxypregn-4-en-3-one (20α-dihydroprogesterone) which in turn can be reduced by a Δ^4-5α-reductase to 20α-hydroxy-5α-pregnan-3-one. A final conversion to 5α,3α,20α-diol is catalyzed by 3α-hydroxysteroid-dehydrogenase. It has been recently demonstrated that brain conversion of progesterone to 3α,5α-P occurs in neurons and astrocytes (Kabbadj et al. 1993). Indeed, the anxiolytic effect of progesterone in animal models is now known to be mediated by GRC-active metabolites of progesterone (Bitran et al. 1995). Collectively, these observations have fueled speculation that the GRC-active neurosteroids may play physiological roles as modulators of neuronal excitability under certain physiological conditions such as stress when levels are sufficient to influence GRC function (Paul and Purdy 1992). The in vivo pharmacology of further reduced metabolites of 3α,5α-P and 3α,5β-P have not been investigated in this regard. The present study provides preliminary evidence that the 20-reduced analogues of 3α,5α-P and 3α,5β-P have anxiolytic activity in two rodent models of anxiety.

The Vogel test is a relatively simple behavioral test for detecting anticonflict properties of drugs (Vogel et al. 1971). Its validity in our hands was supported in part by the observation that diazepam produced a significant increase in punished responding whereas the GRC-inactive neurosteroid 3β,5α-P did not. The ICV route of administration was chosen for the initial comparative studies between the pregnanediols because of certain advantages. First, ICV administration maximizes the amount of active steroid at the site of action since all of the steroids tested have hydroxyl groups in position 3 which render them highly susceptible to rapid liver metabolism (i.e., rapid conjugation and excretion). Second, these neuroactivesteroids have very low water solubility, and thus would be insoluble at the doses required for systemic administration. Third, ICV injection allows detection of drug effects using relatively small quantities of drug when compared to the amounts required for systemic administration. Nevertheless, some of the steroids tested have already been shown to have anxiolytic activity when systemically administered (Wieland et al. 1991, 1995).

Both 3α,5β-P and 3α,5α-P increased punished drinking with greater effect than diazepam in the Vogel test. 3α,5β-P was equipotent to diazepam based on apparent MEDs. The steroid effect observed was stereospecific in that 3β,5α-P, the GRC-inactive steroid, did not produce any statistically significant anticonflict effect at doses up to 100 μg. This is consistent with the relative anxiolytic potency and efficacy of these epimers previously reported for other animal models of anxiety (Bitran et al. 1991). The four endogenously occurring pregnanediols were also active but less potent than their precursors in enhancing punished drinking with MEDs in the range of 10–20 μg. Systemically, 5α,3α,20α-diol was also active in the mouse elevated plus-maze, a nonpunished conflict model of anxiety. This particular pregnanediol was chosen out of the four evaluated in the Vogel test because it has the greatest in vitro potency and is thus the most likely to yield an unequivocal response upon systemic administration. It is possible that the effects of the pregnanediols at the GRC are mediated by back conversion to their respective ketones. However, the latency to onset of action of these steroids in other functional assays (e.g., synaptosomal $^{36}Cl^-$ uptake and GABA-gated Cl^- channel conductance as measured by patch clamp methods) is in the second to millisecond time frame which renders such a possibility unlikely if the hypothesis that their anxiolytic effects are mediated by the GRC proves to be cogent (Peters et al. 1988; Belelli et al. 1989).

In conclusion, preliminary behavioral data indicate that both the 5-reduced-pregnan-3α-ol-20-one steroids and their corresponding pregnanediols are capable of producing robust anxiolytic effects in the models tested. Under physiological conditions, plasma levels of 5α,3α,20α-diol are in the 75 nM range (Ichikawa et al. 1974) during estrus and early diestrus phases of the rat estrous cycle. When compared to the apparent IC_{50} value of 69 nM in vitro as a modulator of [^{35}S]TBPS binding to the GRC (McCauley et al. 1995), it is plausible that physiological levels of 5α,3α,20α-diol alone are sufficient to potentiate GABA-action on chloride channel conductance. These data suggest that if sufficient levels of both the 5-reduced-pregnan-3α-ol-20-one steroids and their further-reduced metabolites are achieved in the brain, they may play an anxiolytic role mediated by the GRC.

Acknowledgement This work was supported by a grant from CoCensys Inc..

References

Arafat ES, Hargrove JT, Maxon WS, Desiderio DM, Wentz AC, Andersen RN (1988) Sedative and hynotic effects of oral

administration of micronized progesterone may be mediated through its metabolites. Am J Obstet Gynecol 159:1203–1209

Belelli D, Gee KW (1989) 5α-Pregnan-3α,20α-diol behaves like a partial agonist in the modulation of GABA-stimulated chloride ion uptake by synaptoneurosomes. Eur J Pharmacol 167: 173–176

Bitran D, Hilvers RJ, Kellogg CK (1991) Anxiolytic effects of 3α-hydroxy-5α-[β]-pregnan-20-one: endogenous metabolites of progesterone that are active at the $GABA_A$ receptor. Brain Res 561:157–161

Bitran D, Shiekh M, McLeod M (1995) Anxiolytic effect of progesterone is mediated by the neurosteroid allopregnanolone at brain $GABA_A$ receptors. J Neuroendocrinol 7:171–177

Corpechot C, Young J, Calvel M, Wehrey C, Veltz JN, Touyer G, Mouren M, Prasad VVK, Banner C, Sjovall J, Baulieu EE, Robel P (1993) Neurosteroids: 3α-hydroxy-5α-pregnan-20-one and its precursors in the brain, plasma and steroidogenic glands of male and female rats. Endocrinology 133:1003–1009

Gee KW, Brinton RE, Chang WC, McEwen BS (1987) Gamma-aminobutyric acid-dependent modulation of the chloride ionophore by steroids in rat brain. Eur J Pharmacol 136: 419–423

Gee KW, Bolger MB, Brinton RE, Coirini H, McEwen BS (1988) Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action. J Pharmacol Exp Ther 246:803–812

Gee KW, McCauley LD, Lan NC (1995) A putative receptor for neurosteroids on the $GABA_A$ receptor complex: pharmacological properties and therapeutic potential of epalons. Crit Rev Neurobiol 9:207–227

Harrison NL, Majewska MD, Harrington JW, Barker JL (1987) Structure-activity relationships for steroid interaction with the $GABA_A$ receptor complex. J Pharmacol Exp Ther 241:346–353

Ichikawa S, Sawada T, Nakamura Y, Morioka H (1974) Ovarian secretion of pregnane compounds during the estrous cycle and pregnancy in rats. Endocrinology 94:1615–1620

Kabbadj K, El Etr M, Baulieu EE, Robel P (1993) Pregnenolone metabolism in rodent embryonic neurons and astrocytes. Glia 7:170–175

Karavolas HJ, Bertics PJ, Hodges D, Rudie N (1984) Progesterone processing by neuroendocrine structures. In: Celotti F, Naftolin F, Martini L (eds) Metabolism of hormonal steroids in the neuroendocrine structures. Sarano Symp. Publications, Raven Press, New York, pp 149–170

Lan NC, Chen JS, Belelli D, Pritchett D, Seeburg PH, Gee KW (1990) A steroid recognition site is functionally coupled to an expressed $GABA_A$-benzodiazepine receptor. Eur J Pharmacol [Mol Pharmacol Sect] 188:403–406

Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM (1986) Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science 232:1004–1007

McCauley LD, Liu V, Hawkinson JE, Lan NC, Gee KW (1995) Selective actions of certain neuroactive pregnanediols at the $GABA_A$ receptor complex in rat brain. Mol Pharmacol 47: 354–362

Paul SM, Purdy RH (1992) Neuroactive steroids. FASEB J 6: 2311–2322

Paxinos G, Watson C (1986) The rat brain stereotaxic coordinates, 2nd edn. Academic Press, Orlando, Fla

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Meth 14:149–167

Peters JA, Kirkness EF, Callachan H, Lambert JJ, Turner AJ (1988) Modulation of the $GABA_A$ receptor by depressant barbiturates and pregnane steroids. Br J Pharmacol 94:1257–1269

Puia G, Santi MR, Vicini S, Pritchett DB, Purdy RH, Paul SM, Seeburg PH, Costa E (1990) Neurosteroids act on recombinant human $GABA_A$ receptors. Neuron 4:759–765

Rodriguez-Sierra JF, Howard JL, Pollard GT, Hendricks SE (1984) Effect of ovarian hormones on conflict behavior. Psychoneuroendocrinology 9:293–300

Vogel JR, Beer B, Clody DE (1971) A simple and reliable conflict procedure for testing anti-anxiety agents. Psychopharmacologia 21:1–7

Wieland S, Lan NC, Mirasadeghi S, Gee KW (1991) Anxiolytic activity of the progesterone metabolite 5α-pregnan-3α-ol-20-one. Brain Res 565:263–268

Wieland S, Belluzzi JD, Stein L, Lan NC (1995) Comparative behavioral characterization of the neuroactive steroids 3α-OH, 5α-pregnan-20-one and 3α-OH, 5β-pregnan-20-one. Psychopharmacology 118:65–71