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Pregnenolone and Pregnenolone Sulfate, Alone and With Ethanol, in Mice on the Plus-Maze

C. L. MELCHIOR*†[1] AND R. F. RITZMANN*

*Department of Psychiatry, Olive View/UCLA Medical Center, Sylmar, CA 91342
†Brentwood Research, West Los Angeles Veterans Administration Medical Center, Los Angeles, CA 90073

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MELCHIOR, C. L. AND R. F. RITZMANN. *Pregnenolone and pregnenolone sulfate, alone and with ethanol, in mice on the plus-maze.* PHARMACOL BIOCHEM BEHAV 48(4) 893–897, 1994. – The neurosteroids pregnenolone and pregnenolone sulfate were tested for anxiogenic/anxiolytic effects in mice on the elevated plus-maze. Pregnenolone in a dose of 0.01 μg/kg increased motor activity and caused an anxiogenic response, i.e., a decreased number of entries onto the open arms of the plus-maze. Pregnenolone sulfate had no effect on motor activity in the doses tested but showed a biphasic response on the plus-maze: at 10.0 and 1.0 μg/kg pregnenolone sulfate caused an anxiogenic response but at 0.1 μg/kg it produced an anxiolytic response. When administered with 1.5 g/kg ethanol, neither neurosteroid altered the depression in motor activity caused by ethanol. However, all doses of pregnenolone tested blocked the anxiolytic effect of ethanol on the plus-maze while one dose of pregnenolone sulfate, 1.0 μg/kg, attenuated the response to ethanol. These results support the suggestion that these neurosteroids could play a role in the initial response to stress and indicate that further work needs to be done to determine the mechanism for the interaction with ethanol.

Neurosteroids Anxiety Pregnenolone Pregnenolone sulfate Ethanol GABA

IT HAS been suggested (21,22) that neurosteroids may contribute to a variety of behaviors or mood states. With knowledge that neurosteroids act at the GABA-benzodiazepine-chloride receptor complex, it is, of course, reasonable to suspect that they could play a role in stress and potentially have antianxiety effects. Several studies have recently supported this idea, showing that neurosteroids that demonstrate GABA agonist properties in biochemical assays have anxiolytic effects in a variety of behavioral tests (1,5–7,32,39). Upon finding that some of these steroids are increased during stress, Purdy et al. (32) suggested they form a novel feedback loop for decreasing the enhanced activity of the hypothalamic-pituitary-adrenal axis after stress.

In the exploration of the biochemical effects of neurosteroids, one compound, pregnenolone sulfate, was described as an antagonist at the GABA receptor, although it does show some biphasic effects (11,23,24). In electrophysiological experiments, several investigators have noted that pregnenolone sulfate reduces the amplitude of responses to GABA (12, 27,28) in a variety of preparations. In membrane patches of rat cortical neurons in primary cultures, Mienville and Vicini (27) demonstrated that both the neurosteroid pregnenolone sulfate and the GABA antagonist picrotoxin selectively decreased the opening frequency of the GABA activated chloride channels.

In tests of memory in mice, Flood and his colleagues (9) demonstrated dose–response curves for both pregnenolone sulfate and its parent compound, pregnenolone. The maximum improvement in memory occurred at very low doses, indicating that a low dose range of these neurosteroids has interesting behavioral effects. These behavioral effects are consistant with GABA antagonist activity (8).

In reviewing the neurochemistry of pregnenolone sulfate, Majewska (21,22) concluded that this excitatory compound could play a role in the initial arousal phase of the stress response. However, there have as yet been no reports of behavioral effects of this nature.

As with the neurosteroids, many of the effects of ethanol

[1] Requests for reprints should be addressed to Dr. C. L. Melchior, Department of Psychiatry, Olive View/UCLA Medical Center, 14445 Olive View Drive, Sylmar, CA 91342-1495.

are thought to be mediated by the potentiation of $GABA_A$ receptor function (14,18,35,36). Given that ethanol and neurosteroids both act on the GABA system, one would expect an interaction between the neurosteroids and ethanol. We have shown that a GABA agonist steroid, pregnanolone, enhances the depressant effects of ethanol (25). In contrast, previous studies of high doses of pregnenolone sulfate have demonstrated little interaction with high (anesthetic) doses of ethanol (3,25). Recently, Korneyev et al. (17) reported that an anesthetic dose of ethanol produced an increase in brain levels of pregnenolone and progesterone in rats, but this did not contribute to the anesthetic actions of ethanol. In contrast, earlier, preliminary studies indicated that a moderate dose of ethanol did not alter pregnenolone and pregnenolone sulfate levels in rat brain (33,38).

The purpose of the current study is to explore the behavioral effects of low doses of pregnenolone sulfate and its parent compound, pregnenolone, alone and with ethanol in a test of anxiety, the elevated plus-maze. These experiments directly address the question of whether or not these neurosteroids can enhance anxiety and, thus, support their possible role in the initial phase of a stress reaction, and explore their interaction with an anxiolytic agent, ethanol. Because the interaction of ethanol with the GABA receptor is different at lower than higher doses (14), a low dose of this drug, in the range that is anxiolytic, might have a different interaction with these neurosteroids than a high dose.

METHOD

Subjects

Male C57BL/6 mice obtained from NCI weighing 20–25 g were used. The animals were housed five per cage in temperature (22 ± 1°C)- and light (0600–1800 h)-controlled rooms. Food (Purina Laboratory Chow) and water were available ad lib. Mice were housed in the animal facility for at least 7 days prior to the initiation of any experiment.

Drugs

Pregnenolone and pregnenolone sulfate (Sigma Chemical Co., St. Louis, MO) were prepared daily as a suspension in a vehicle of saline containing 0.4% Tween 80. All intraperitoneal neurosteroid injections were made at a volume of 0.1 cc/10 g b.wt. Control subjects received a similar injection of vehicle. Ethanol was prepared for intraperitoneal injection as a 20% w/v solution from 95% ethanol and saline. Saline was administered in place of ethanol as a control in the neurosteroid alone groups.

Procedure

Pregnenolone and pregnenolone sulfate were examined for their effects on two tests. Locomotor activity was assessed initially to determine whether sedation or hyperactivity occurred. This was followed by a test for anxiety, the elevated plus-maze (19,26).

To evaluate the effect of the neurosteroids on motor activity an individual animal was placed in a large plastic cage (25 × 48 × 16 cm, W × L × H), which was placed on a platform of an activity monitor (Stoelting Instruments, Columbus, OH). After a 20-min acclimation period, subjects were injected with a neurosteroid or a neurosteroid plus ethanol and activity was recorded for 20 min.

Upon completing the activity measure, the animal was tested on the elevated plus-maze (19,26). The elevated plus-maze is made of black Plexiglas consisting of two opposite-facing open arms (30 × 5 cm) and two opposite-facing closed arms (30 × 5 × 15 cm). The walls of the closed arms are clear Plexiglas and are separated by a central area which is 5 × 5 cm. The whole plus-maze is mounted on a base, raising it 38 cm above the floor. Testing involves placing a mouse at one end of one of the open arms. The time the mouse takes to

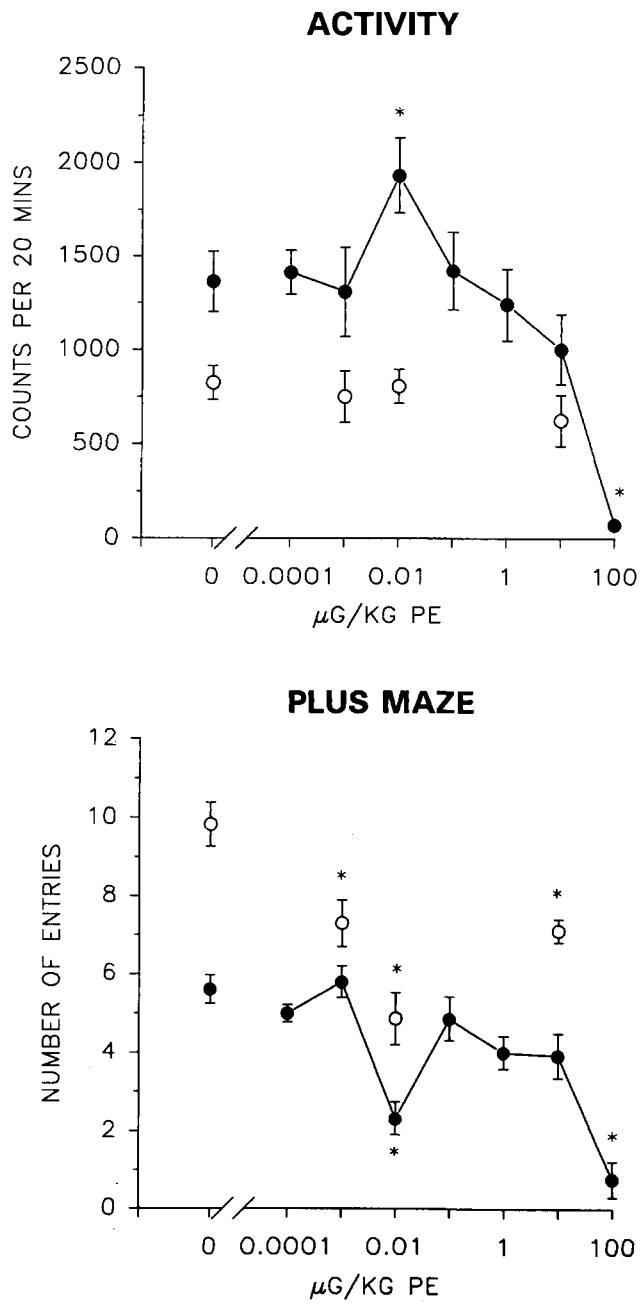

FIG. 1. A dose-response curve for pregnenolone (PE) alone (solid circles) or with 1.5 g/kg ethanol (open circles) in mice on an activity monitor (top) or on the elevated plus maze (bottom). n = 7–14 per group except for 0 μg/kg PE alone group in which n = 20. *p < 0.05, Dunnett test.

leave the start position (the first 10 cm of the open arm) is recorded (start time). The time it takes until the mouse enters half way into one of the closed arms is also recorded (run time). At this time the 3-min test session was begun. The number of times the mouse enters onto the open arms was recorded. An entry was defined as placing at least two paws onto the open arm. This modified procedure has been used previously for testing C57BL/6 mice (26).

Statistical Analysis

Data for a given test was subjected to analysis of variance followed by a Dunnett test. Specific paired comparisons were done with a Student's *t*-test. Statistically significant differences were noted at the 0.05 level. Because all testing could not be done on one day, at least two doses of a neurosteroid plus vehicle controls were run on a given day. Data for each group was comprised of animals tested on at least 2 separate days. There were 7–14 mice used in each group except for the vehicle controls, which did not differ over days and were pooled for an $n = 20$. Each mouse was tested only once.

RESULTS

Pregnenolone

Motor activity. As shown in Fig. 1, top, a high dose of pregnenolone, 100 μg/kg, suppressed motor activity, whereas a lower dose, 0.01 μg/kg, increased activity above the level of the vehicle controls.

At 1.5 g/kg, ethanol by itself decreased motor activity, $t(21) = 2.827, p < 0.05$. Pregnenolone did not influence this effect of ethanol.

Plus-maze. On the plus-maze (Fig. 1, bottom), 100 μg/kg pregnenolone decreased the number of open arm entries, probably because of its effect on motor activity. However, at 0.01 μg/kg, a decrease was again observed.

Ethanol by itself, at 1.5 g/kg, increased the number of open arm entries, $t(29) = 8.286, p < 0.05$. All doses of pregnenolone given with ethanol blocked this response.

Pregnenolone by itself did not effect start time, $F(7, 66) = 0.91$, nor did it alter run time, $F(7, 66) = 2.00$. Pregnenolone, in combination with ethanol, also did not alter start time, $F(3, 61) = 1.69$, nor run time, $F(3, 61) = 2.34$.

Pregnenolone Sulfate

Motor activity. Figure 2, top, shows that pregnenolone sulfate had no effect on motor activity in the dose range tested. Given with 1.5 g/kg ethanol, pregnenolone sulfate had no effect on the decrease in activity caused by ethanol.

Plus-maze. Pregnenolone sulfate exhibited a biphasic dose response curve on the plus-maze (Fig. 2, bottom), decreasing the number of open arm entries on the plus-maze at doses of 10.0 μg/kg and 1.0 μg/kg, and increasing the number of open arm entries at 0.1 μg/kg.

As noted earlier, a dose of 1.5 g/kg ethanol increased the number of open arm entries, $t(22) = 7.967, p < 0.05$. This effect was attenuated only by the 1.0 μg/kg dose of pregnenolone sulfate.

Pregnenolone sulfate did not alter start time, $F(5, 55) = 1.15$, nor run time, $F(5, 55) = 2.05$. Pregnenolone sulfate, in combination with ethanol, also did not alter start time, $F(5, 60) = 2.28$, nor run time, $F(5, 60) = 2.02$.

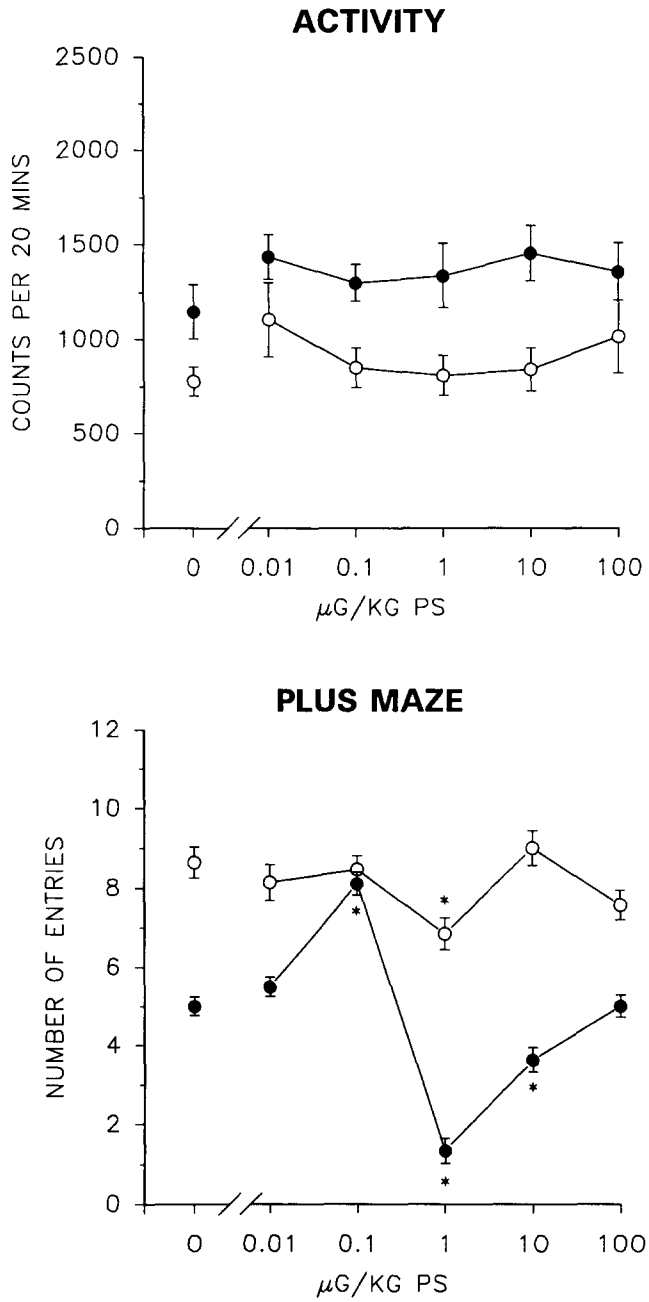

FIG. 2. A dose–response curve for pregnenolone sulfate (PS) alone (solid circles) or with 1.5 g/kg ethanol (open circles) in mice on an activity monitor (top) or on the elevated plus maze (bottom). $n = 8$–13 per group. $^*p < 0.05$, Dunnett test.

DISCUSSION

This study shows that both pregnenolone and pregnenolone sulfate have interesting behavioral effects at exceedingly low doses. Pregnenolone, at 0.01 μg/kg, increases anxiety (as reflected in the behavior on the plus-maze). Pregnenolone sulfate, depending on the dose, can be either anxiogenic (10.0 or 1.0 μg/kg) or anxiolytic (0.1 μg/kg). In examining the interaction with ethanol, pregnenolone sulfate attenuated the anxiolytic effect of ethanol on the plus-maze at one dose. In con-

trast, pregnenolone blocked the anxiolytic effects of ethanol on the plus-maze in a wide range of doses (0.001–10.0 μg/kg).

The mixed agonist/antagonist effect of pregnenolone sulfate on the receptors associated with the $GABA_A$ receptor complex (22) could account for the biphasic dose–response curve seen in the plus-maze data. The GABA antagonist properties of the neurosteroid could explain the attenuation of the effect of ethanol. However, the recently reported effects of pregnenolone sulfate as a positive allosteric modulator at the NMDA receptor complex (4,15,20,40) could also contribute to the anxiogenic responses observed with pregnenolone sulfate alone. Because ethanol has been shown to inhibit the actions of NMDA agonists (13), the limited influence of pregnenolone sulfate on the effects of ethanol would be consistent with the actions of the drugs in this system.

Much less has been reported on the neurochemical activity of pregnenolone than pregnenolone sulfate. Pregnenolone has not shown activity in binding assays relative to the receptors associated with the GABA receptor complex (10,31,37). The one neurochemical effect reported for pregnenolone, as well as pregnenolone sulfate, is an inhibition of voltage gaited Ca^{2+} current in hippocampal cells (34). However, there is evidence that calcium channel inhibitors have a modest anxiolytic effect (30), and the calcium channel antagonist nifedipine has been reported to be ineffective in influencing the anxiolytic action of ethanol in the elevated plus-maze in rats (16). Therefore, an action on voltage gaited calcium channels is not a likely mechanism to explain the data obtained here. Clearly, a further exploration of the actions of pregnenolone is required to determine the mechanism by which it blocks the effects of ethanol.

Pregnenolone is a steroid which is an initial step in a metabolic pathway that leads to the production of other compounds that have anxiolytic activity (2,26,29,33), as well as pregnenolone sulfate, which this study indicates has biphasic effects. In considering the suggestion that neurosteroids play a role in stress, these results support the speculation that pregnenolone and higher levels of pregnenolone sulfate, which are anxiogenic, participate in the initial response to stressful stimuli, which is then terminated by the action of lower levels of pregnenolone sulfate and the other metabolites that are anxiolytic (21,22,32).

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REFERENCES

1. Bitran, D.; Hilvers, R. J.; Kellogg, C. K. Anxiolytic effects of 3a-hydroxy-5a[β]-pregnan-20-one-Endogenous metabolites of progesterone that are active at the $GABA_A$ receptor. Brain Res. 561: 157–161; 1991.
2. Bitran, D.; Purdy, R. H.; Kellogg, C. K. Anxiolytic effect of progesterone is associated with increases in cortical allopregnanolone and $GABA_A$ receptor function. Pharmacol. Biochem. Behav. 45:423–428; 1993.
3. Bowers, B. J.; Wehner, J. M. Biochemical and behavioral effects of steroids on $GABA_A$ receptor function in Long-Sleep and Short-Sleep mice. Brain Res. Bull. 29:57–68; 1992.
4. Bowlby, M. R. Pregnenolone sulfate potentiation of *N*-methyl-D-aspartate receptor channels in hippocampal neurons. Mol. Pharmacol. 43:813–819; 1993.
5. Britton, K. T.; McLeod, S.; Koob, G. F.; Hauger, R. Pregnane steroid alphaxalone attenuates anxiogenic behavioral effects of corticotropin releasing factor and stress. Pharmacol. Biochem. Behav. 41:399–403; 1992.
6. Britton, K. T.; Page, M.; Baldwin, H.; Koob, G. F. Anxiolytic activity of steroid anesthetic alphaxalone. J. Pharmacol. Exp. Ther. 258:124–129; 1991.
7. Crawley, J. N.; Glowa, J. R.; Majewska, M. D.; Paul, S. M. Anxiolytic activity of an endogenous adrenal steroid. Brain Res. 398:382–385; 1986.
8. Decker, M. W.; McGaugh, J. L. The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory. Synapse 7:151–168; 1991.
9. Flood, J. F.; Morley, J. E.; Roberts, E. Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. Proc. Natl. Acad. Sci. USA 89:1567–1571; 1992.
10. Gee, K. W.; Bolger, M. B.; Brinton, R. E.; Coirini, H.; McEwen, B. S. 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; 1988.
11. Gee, K. W.; Joy, D. S.; Belelli, D. Complex interactions between pregnenolone sulfate and the *t*-butylbicyclophosphorothionate-labeled chloride ionophore in rat brain. Brain Res. 482:169–173; 1989.
12. Harrison, N. L.; Majewska, M. D.; Harrington, J. W.; Barker, J. L. Structure-activity relationships for steroid interaction with the gamma aminobutyric $acid_A$ receptor complex. J. Pharmacol. Exp. Ther. 241:346–353; 1987.
13. Hoffman, P. L.; Rabe, C. S.; Grant, K. A.; Valverius, P.; Hudspith, M.; Tabakoff, B. Ethanol and the NMDA receptor. Alcohol 7:229–231; 1990.
14. Huidobro-Toro, J. P.; Bleck, V.; Allan, A. M.; Harris, R. A. Neurochemical actions of anesthetic drugs on the gamma-aminobutyric acid receptor-chloride channel complex. J. Pharmacol. Exp. Ther. 242:963–969; 1987.
15. Irwin, R. P.; Maragakis, N. J.; Rogawski, M. A.; Purdy, R. H.; Farb, D. H.; Paul, S. M. Pregnenolone sulfate augments NMDA receptor mediated increases in intracellular Ca^{2+} in cultured rat hippocampal neurons. Neurosci. Lett. 141:30–34; 1992.
16. Kirac, R.; Eroglu, L. Bay K-8644 Potentiates the anxiolytic effect of ethanol. Pharmacol. Biochem. Behav. 39:325–327; 1991.
17. Korneyev, A. Y.; Costa, E.; Guidotti, A. During anesthetic-induced activation of hypothalamic pituitary adrenal axis, blood-borne steroids fail to contribute to the anesthetic effect. Neuroendocrinology 57:559–565; 1993.
18. Leidenheimer, N. J.; Harris, R. A. Acute effects of ethanol on $GABA_A$ receptor function: Molecular and physiological determinants. In: Biggio, G.; Concas, A.; Costa, E., eds. GABAergic synaptic transmission. New York: Raven Press; 1992:269–279.
19. Lister, R. G. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berlin) 92:180–185; 1987.
20. Maione, S.; Berrino, L.; Vitagliano, S.; Leyva, J.; Rossi, F. Pregnenolone sulfate increases the convulsant potency of *N*-methyl-D-aspartate in mice. Eur. J. Pharmacol. 219:477–479; 1992.
21. Majewska, M. D. Actions of steroids on neuron: Role in personality, mood, stress, and disease. Integr. Psychiatry 5:258–273; 1987.
22. Majewska, M. D. Neurosteroids—Endogenous bimodal modulators of the $GABA_A$ receptor—Mechanism of action and physiological significance. Prog. Neurobiol. 38:379–395; 1992.
23. Majewska, M. D.; Demirgoren, S.; London, E. D. Binding of pregnenolone sulfate to rat brain membranes suggests multiple sites of steroid action at the $GABA_A$ receptor. Eur. J. Pharmacol. 189:307–315; 1990.
24. Majewska, M. D.; Schwartz, R. D. Pregnenolone sulfate: An endogenous antagonist of the gamma-aminobutyric acid receptor complex in brain? Brain Res. 404:355–360; 1987.
25. Melchior, C. L.; Allen, P. M. Interaction of pregnanolone and pregnenolone sulfate with ethanol and pentobarbital. Pharmacol. Biochem. Behav. 42:605–611; 1992.

26. Melchior, C. L.; Ritzmann, R. F. Dehydroepiandrosterone is an anxiolytic in mice on the plus-maze. Pharmacol. Biochem. Behav. 47:437–441; 1994.
27. Mienville, J.-M.; Vicini, S. Pregnenolone sulfate antagonizes $GABA_A$ receptor-mediated currents via a reduction of channel opening frequency. Brain Res. 489:190–194; 1989.
28. Ong, J.; Kerr, D. I. B.; Johnston, G. A. R. Pregnenolone and its sulphate modulate $GABA_A$-receptor-mediated contractile responses in the guinea-pig isolated ileum. Eur. J. Pharmacol. 142: 461–464; 1987.
29. Paul, S. M.; Purdy, R. H. Neuroactive steroids. FASEB J. 6: 2311–2322; 1992.
30. Pucilowski, O. Psychopharmacological properties of calcium channel inhibitors. Psychopharmacology (Berlin) 109:12–29; 1992.
31. Purdy, R. H.; Morrow, A. L.; Blinn, J. R.; Paul, S. M. Synthesis, metabolism, and pharmacological activity of 3-alpha-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. J. Med. Chem. 33:1572–1581; 1990.
32. Purdy, R. H.; Morrow, A. L.; Moore, P. H.; Paul, S. M. Stress-induced elevations of gamma-Aminobutyric Acid type-A receptor-active steroids in the rat brain. Proc. Natl. Acad. Sci. USA 88:4553–4557; 1991.
33. Robel, P.; Akwa, Y.; Corpechot, C.; Hu, Zhong-Y.; Jung-Testas, I.; Kabbadj, K.; Le Goascogne, C.; Morfin, R.; Vourc'h, C.; Young, J.; Baulieu, E.-E. Neurosteroids: Biosynthesis and function of pregnenolone and dehydroepiandrosterone in the brain. In: Motta, M., ed. Brain endocrinology. 2nd ed. New York: Raven Press; 1991:105–132.
34. Spence, K. T.; Plata-Salaman, C. R.; Ffrenchmullen, J. M. H. The neurosteroids pregnenolone and pregnenolone-sulfate but not progesterone, block Ca^{2+} currents in acutely isolated hippocampal CA1 neurons. Life Sci. 49:PL235–PL239; 1991.
35. Ticku, M. K.; Burch, T. P.; Davis, W. C. The interactions of ethanol with the benzodiazepine-GABA receptor-ionophore complex. Pharmacol. Biochem. Behav. 18(Suppl. 1):15–18; 1983.
36. Ticku, M. K.; Kulkarni, S. K. Molecular interactions of ethanol with GABAergic system and potential of RO 15-4513 as an ethanol antagonist. Pharmacol. Biochem. Behav. 30:501–510; 1988.
37. Turner, D. M.; Ransom, R. W.; Yang, J. S.-J.; Olsen, R. W. Steroid anesthetics and naturally occuring analogs modulate the gamma-aminobutyric acid receptor complex at a site distinct from barbiturates. J. Pharmacol. Exp. Ther. 248:960–966; 1989.
38. Vatier, O. C.; Bloom, F. E. Effect of ethanol on 3 beta-hydroxy-delta 5 steroid concentration in the rat brain. Alcohol.: Clin. Exp. Res. 12:316; 1988.
39. Wieland, S.; Lan, N. C.; Mirasedeghi, S.; Gee, K. W. Anxiolytic activity of the progesterone metabolite 5alpha-pregnan-3alpha-ol-20-one. Brain Res. 565:263–268; 1991.
40. Wu, F. S.; Gibbs, T. T.; Farb, D. H. Pregnenolone sulfate—A positive allosteric modulator at the *N*-methyl-D-aspartate receptor. Mol. Pharmacol. 40:333–336; 1991.