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EVALUATION OF THE ANALGESIC EFFECT OF NEUROSTEROIDS AND THEIR POSSIBLE MECHANISM OF ACTION

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Abstract : The present study investigates the effect of progesterone (P), a pregnane precursor of neurosteroids and 4-chlordiazepam (4-CD), a high affinity ligand for mitochondrial diazepam binding inhibitor receptor (MDR) that stimulates neurosteroid synthesis, in both acute, (tail flick latency test, TFL) and chronic, (formalin-induced pain response, FT), models. Both P and 4-CD showed an analgesic response in these models. The effect of P and 4-CD was antagonized by bicuculline on TEL but not in FT. However, naloxone attenuated the antinociceptive response of P and 4-CD in TFL as well as FT. Further, P and 4-CD pretreatment potentiated the analgesic effect of morphine and nimodipine in both the models of pain sensitivity. Thus, neurosteroids produce an antinociceptive effect which may be mediated by modulation of GABAergic and/or opioidergic mechanisms as well as voltage gated calcium channels.

Key words : neurosteroids, analgesic response, formalin test, tail flick latency test

INTRODUCTION

It is now becoming evident that some of the steroids like progesterone, besides producing endocrine effects on peripheral tissues also target brain for a number of neuroendocrine and behavioral effects. Recent studies have demonstrated that the enzymes involved in the biosynthesis of some of these steroidal hormones are found in cell-specific areas in the brain. These brain derived steroids are called 'Neurosteroids' (1). Like, in peripheral tissues they are the derivatives of cholesterol and synthesized in the glial cells. Some of the important neurosteroids are progesterone (P), allopregnanolone (AP), 5∝-pregnan-3∝-21-diol-20-one (THDOC), pregnenolone sulphate (PS) and dehydroepiandrosterone (DHEA).

The concept that neurosteroids are synthesized locally in the brain was confirmed for the first time in 1987 (2) and since then there has been considerable research efforts to find out their physiological role and mechanism(s) of action. Biochemical, electrophysiological and behavioral evidence suggest a neuromodulatory role of these

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compounds especially allosteric bimodal modulation of GABA-A receptor chloride channels (3), inhibition of glycine chloride channels (4) and voltage gated calcium channels (VGCCs) (5) and potentiation of N-methyl-D-aspartate (NMDA) receptor responses (6).

Recently a pathway for neurosteroidogenesis has been delineated in the brain and a role of mitochondrial diazepam binding inhibitor receptor (MDR) in the neurosteroid biosynthesis has been demonstrated (7). Activation of MDRs by appropriate ligand facilitates the intramitochondrial flux of cholesterol and thereby increases the availability of cholesterol to cytochrome P450 side chain cleavage ($P450_{scc}$) enzyme that catalyses the cholesterol side chain cleavage to yield pregnenolone (7). One such MDR ligand is 4-chlordiazepam (4-CD).

Many of these neurosteroids exhibit antistress, anxiolytic and cognition enhancing activities (8). P has been reported to produce antinociceptive effect in an acute pain model (9), but its role in chronic pain models is not known. Moreover, the mechanisms involved in mediating the analgesic response are also needed to be evaluated. Keeping this in view in the present study the effect of P and 4-CD was investigated in both acute, (tail flick latency test, TFL) and chronic, (formalin-induced pain response, FT), models of pain. Furthermore, their interactions with GABAergic and opioidergic agents and calcium channel blockers (CCBs) were studied to explore their mechanism(s) of actions.

METHODS

Animals

The study was carried out in Swiss male mice weighing 25-30 g. The animals were procured from the Central Animals House of the Institution and housed in temperature (22±2° C) and light (12 h light-12 h dark cycle) controlled conditions. Pellet diet (Brooke Bond Lipton, India Ltd.) and water were available ad *libitum*, except 1h before and during the experiment. The animals were maintained as per the "Guidelines for the Care and Use of Animals in Scientific Research" prepared by the Indian National Science Academy, New Delhi (10).

Drugs and chemicals

Progesterone (P). 4-Chlordiazepam (4-CD), Naloxone and Bicuculline (Sigma Chemical Co., U.S.A.); Nimodipine (Cipla Ltd.) and Morphine sulfate were used in the study. P and 4-CD were dispersed in 1% Tween 80 and diluted with saline, nimodipine was dissolved in 100% ethanol and morphine sulfate, naloxone and bicuculline were dissolved in distilled water. Formalin (0.5%) (v/v) solution was prepared by adding normal saline to the stock solution of 4% formaldehyde in water.

Treatment schedule

Animals were randomly allocated into different groups, each group comprising of 8-10 mice. P and 4-CD were injected subcutaneously (s.c.) and morphine, bicuculline and nimodipine intraperitoneally

(i.p.) 30 min, whereas naloxone was administered i.p. 15 min, before performing the analgesic test. The dose and the time intervals of drugs were selected on the basis of the results of pilot experiments. All experiments were performed between 10.00 a.m.-3.00 p.m. Respective vehicle groups were used with different drugs. Since the data from various vehicle groups were not significantly different, data from different groups were pooled and presented.

Formalin Test (FT) : The test was performed by injecting 0.2 ml of 0.5% formalin s.c. under the plantar surface of right hind paw of the mouse (11). Left paw was injected with 0.2 ml of 0.9% saline and acted as control. Two distinct periods of intense licking of the right paw were observed after administrating formalin, i.e. an early phase lasting from 0-5 min and a late phase during 25-30 min. These two periods were scored separately for recording drug effect. For each phase licking response was recorded for 5 min. There was no licking response observed in the control paw.

Tail Flick Latency Test (TFT) : The method of Davies et al (12) was modified as follows: The mouse was restrained and its tail was placed 2mm above the heated wire of analgesiometer (TECHNO). The temperature to which the tail was exposed ranged between 50-52°C. A cut off time of 4 sec was taken, i.e. animals with reaction time of more than 4 sec were not used for the study. A cut off time of 10 sec was used for drug studies, i.e. if the mouse did not respond in 10 sec, it was removed from the heat source and given a score of 10 sec.

Statistical analyses

All results are expressed as mean±S.E.M. Data from FT (early and late phases) and TFT were analysed by the Mann Whiteny 'U' test and 'Student's two tailed 't' test, respectively. Difference with 'p' value of less than 0.05 was considered as significant.

RESULTS

Formalin test

P (5, 10 mg/kg s.c.) and 4-CD (0.25, 0.5 mg/kg s.c.) produced an antinociceptive effect on formalin-induced pain response. There was a significant reduction in the time an animal spent in licking the paw both during the early and the late phase (Table 1). The antinociceptive response of both P and 4-CD was effectively blocked by naloxone (1 mg/kg i.p.), an opioid antagonist but not by bicuculline (1 mg/kg i.p.), a GABA-A receptor antagonist (Table II). Nimodipine (20, 40 mg/kg, i.p.) and morphine (2.5, 5 mg/kg i.p.) also decreased the pain sensitivity in both the phases. Pretreatment with P (5mg/kg) or 4-CD (0.25 mg/kg) before morphine and nimodipine potentiated analgesic effect of the latter drugs (Table I).

Tail flick test

Both P (5, 10 mg/kg s.c.) and 4-CD (0.25, 0.5 mg/kg, s.c.) dose dependently increased the duration of tail flick latency (Table I). The analgesic response of these compounds was significantly antagonised by naloxone (1 mg/kg, i.p.) as well as bicuculline (1 mg/kg, i.p.)

TABLE I: Effect of progesterone, 4[1]-chlordiazepam, morphine and nimodipine in formalin induced pain response and tail flick latency test.

Treatment (mg/kg)	*#Licking response (sec) (Mean±SEM)*		*†Tail flick latency (sec) (Mean±SEM)*
	Early phase (0–5 min)	*Late phase (25–30 min)*	
††Vehicle	120.2±8.4	98.5±9.8	2.4±0.3
Progesterone (5)	88.6±10.2*	52.8±11.6*	5.2±0.8**
Progesterone (10)	60.8±6.8**	28.6±6.2**	7.2±0.6**
4[1]-Chlordiazepam (0.25)	92.4±7.4*	44.2±7.8*	5.6±0.5**
4[1]-Chlordiazepam (0.5)	65.2±4.8**	24.4±8.2**	9.4±0.5**
Morphine (2.5)	92.42±10.25*	72.22±12.3*	4.2±1.0**
Morphine (5)	40.42±9.2**	24.0±8.9**	7.8±1.2**
Progesterone (5) + Morphine (2.5)	70.44±8.8*	40.4±7.2*	6.4±2.0*
4[1]-Chlordiazepam (0.25) + Morphine (2.5)	81.0±7.2*	42.4±6.1*	6.2±2.2*
Nimodipine (20)	78.6±10.2*	52.8±13.6**	2.6±0.6
Nimodipine (40)	52.2±12.8**	38.6±8.20**	3.8±0.4
Progesterone (5) + Nimodipine (20)	48.8±6.2[XX]	32.6±7.2[XX]	8.2±1.2[XX]
4[1]-Chlordiazepam (0.25) + Nimodipine (20)	50.4±8.4[XX]	40.2±10.6[XX]	9.6±0.4[XX]

n = 8
#Mann Whitney 'U' test †Student's 't' test
††Data pooled from various vehicle groups
*P<0.05 **P<0.001 vs Vehicle treated (control) group
*P<0.05 vs Morphine (2.5) treated group
[XX]P<0.001 vs Nimodipine (20) treated group

TABLE II: Modulation of the effect of progesterone and 4'-chlordiazepam by naloxone and bicuculline in formalin induced pain response.

Treatment (mg/kg)	*#Licking response (sec) (Mean±SEM)*	
	Early phase (0–5 min)	*Late phase (25–30 min)*
††Vehicle	120.2±8.4	98.5±9.8
Progesterone (10)	60.8±6.8**	28.6±6.2**
Progesterone (10) + Naloxone (1)	112.4±7.2**	90.4±8.5**
Progesterone (10) + Bicuculline (1)	65.2±9.4	30.0±10.2
4[1]-Chlordiazepam (0.5)	65.2±4.8**	24.4±8.2**
4[1]-Chlordiazepam (0.5) + Naloxone (1)	108.6±6.6[XX]	58.2±10.4[XX]
4[1]-Chlordiazepam (0.5) + Bicuculline (1)	70.8±9.8	39.2±11.4

n = 8
#Mann Whitney 'U' test
††Data pooled from various vehicle groups
**P<0.001 vs Vehicle (control) treated group
**P<0.001 vs Progesterone treated group
[XX]P<0.001 vs 4[1]-Chlordiazepam treated group

(Table III). Nimodipine (20,40mg/kg, i.p.) per se did not show any antinociceptive effect in this model of pain sensitivity. However, when administered along with P (5 mg/kg) or 4-CD (0.25 mg/kg) produced a significant increase in the tail flick latency which was found to be far more than the response observed with the same dose of P or 4-CD alone. Morphine (2.5, 5 mg/kg, i.p.) also produced a dose-dependent analgesic response which was potentiated by pretreating the animals with P (5 mg/kg) or 4-CD (0.25 mg/kg) (Table I).

TABLE III : Modulation of the effect of progesterone and 4|-chlordiazepam by naloxone and bicuculline in tail flick latency test.

Treatment (mg/kg)	*#Tail flick latency (sec) (Mean±SEM)*
††Vehicle	2.4±0.3
Progesterone (10)	7.2±0.6**
4|-Chlordiazepam (0.5)	9.4±0.5**
Progesterone (10) + Naloxone (1)	4.0±1.5++
4|-Chlordiazepam (0.5) + Naloxone (1)	3.6±0.8++
Progesterone (10) + Bicuculline (1)	3.0±1.2XX
4|-Chlordiazepam (0.5) + Bicuculline (1)	3.8±1.0XX

n = 8
#Student's 't' test
††Data pooled from various vehicle groups
**P<0.05 vs Vehicle treated (Control) group
++P<0.01 vs Progesterone treated group
XXP<0.001 vs 4|-Chlordiazepam treated group

DISCUSSION

In the present study P, a pregnane precursor of neurosteroids, that is devoid of binding capacity to GABA-A receptors (13) and 4-CD, a high affinity MDR ligand, that potently stimulates mitochondrial neurosteriod synthesis (7) showed a significant analgesic response in both the acute and chronic models of pain, i.e. TFL and FT, respectively.

P is metabolized to AP and THDOC in the neurons and glia (14). Further, 4-CD is reported to facilitate the synthesis of AP and THDOC by activation of MDR (7). These two neurosteroids, i.e. AP and THDOC are the direct agonists of the allosteric modulatory sites located in the transmemberance domain of GABA-A receptors (15). The antinociceptive effect of both these compounds (P and 4-CD), observed in the present study seem to be mediated via GABA-A-chloride channel complex in TFL as this response was nullified by bicuculline, a GABA-A receptor antagonist. However, failure of modulation of the analgesic response of these compounds by bicuculline in FT, suggests the possibility that some other mechanism may also be involved in the antinociceptive action of P and 4-CD.

Neurosteroids have been shown to inhibit VGCCs (5) and various CCBs are reported to exhibit an antinociceptive response (16–18). Hence, analgesic effect of neurosteroids observed in FT may be mediated via inhibition of VGCCs. FT is a model of tonic (countinuous) pain (19). Since L-type calcium channels open in depolarized state (20), the likelihood of their being opened during continuous pain is much more. In the present study, nimodipine, a CCB per se exhibited an antinociceptive effect in FT but failed to do so in TFL. This is in consonance with the earlier observations which reported that various CCBs produced a significant analgesic response in FT but not in TFL (18). Potentiation of the analesic response of nimodipine by pretreating the animals with P or 4-CD suggests that

inhibition of VGCCs may be involved in the antinociceptive effect of neurosteriods. Naloxone, an opioid antagonist blocked the analgesic effect of P and 4-CD on both the models of pain sensitivity used in the present study. Furthe r, the analgesic effect of morphne was potentiatted by both P and 4-CD, suggesting thereby that opioid receptors may also be involved in mediating the analgsic response of neurosteroids. P and other gonadal steriods have been shown to interact with and effect endogenons opioid systems (21). Moreover, opioid receptors have been shown to modulate different types of calcium channels, like the P, Q and L-type calcium channels are inhibited by activation of the mu, delta or kappa opioid receptors (22). Therefore, it is possible that neurosteriods may be exerting antinociceptive effect via modulating opioid receptors linked to calcium channels. Thus, neurosteroids exhibit a significant analgesic response which may be mediated via modulation of GABAergic and/or opioidergic mechanisms and voltage gated calcium channels.

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