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Perispinal Progestins Enhance the Antinociceptive Effects of Muscimol in the Rat

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CABA, M., G. GONZÁLEZ-MARISCAL AND C. BEYER. *Perispinal progestins enhance the antinociceptive effects of muscimol in the rat.* PHARMACOL BIOCHEM BEHAV **47**(1) 177–182, 1994. – The intrathecal (IT) injection of progesterone (PROG) or three of its ring A-reduced metabolites (5β,3α-pregnanolone, 5α,3α-pregnanolone, or 5β,3β-pregnanolone) did not significantly alter any of two pain thresholds (vocalization threshold to tail shock, VTTS, or tail flick latency, TFL) in ovariectomized rats when tested in a wide range of doses (2.5–250 μg). When combined with a subanalgesic dose of muscimol (MUSC; 1 μg IT), PROG and its two 3α-hydroxy derivatives, but not the 3β, caused significant analgesia in the VTTS but not in the TFL test. No clear dose–response relationships were noted in the analgesic response to the combination of the progestins and MUSC. The present results indicate that PROG, either directly or through its ring A-reduction, can modulate nociceptive information by enhancing the action of GABA agonists on $GABA_A$ receptors.

Pain Nociception Analgesia GABA Muscimol Progesterone Progestins Pregnanolones $GABA_A$ receptor Intrathecal Spinal cord

A HIGH concentration of GABAergic neurons and receptors exists in spinal cord regions (laminae I to III) where fibers carrying nociceptive information from the periphery terminate (1,19,22,24). This anatomical arrangement suggests the existence of a spinal GABAergic system modulating pain perception. Additional support for this idea is that the intrathecal (IT) administration of $GABA_A$ and $GABA_B$ agonists induces analgesia (4,10,25,26), while, conversely, $GABA_A$ antagonists (picrotoxin and bicuculline) decrease nociceptive thresholds and induce skin hyperalgesia [allodynia (25)]. Little is known about the factors regulating the activity of this nociceptive inhibitory system. Perispinal administration of the $GABA_A$ antagonist bicuculline blocked the analgesic effect of vaginocervical stimulation in the rat, demonstrating that this system is activated by some types of peripheral stimulation (25).

Several results, coming mainly from in vitro studies, indicate that progestins can enhance the affinity of $GABA_A$ receptors for its agonists (11,12,18,20). Therefore, an increase in the plasma concentration of progesterone (PROG) or its metabolites should facilitate the inhibitory action of some GABAergic systems. Indeed, systemic administration of PROG to ovariectomized rats increases nociceptive thresholds when combined with a subanalgesic dose of IT muscimol [MUSC, a $GABA_A$ agonist (21)]. However, the site and mechanism of action of PROG in this study were uncertain, since the observed analgesia could have been due to either a direct interaction of the steroid with the occupied spinal $GABA_A$ receptor or to the activation of brain regions modulating nociception through descending influences to the spinal cord. These could, in turn, summate their actions to those of the spinal GABAergic system. This last possibility was supported by the observation that the intracerebroventricular (ICV) administration of progestins reduced in the 3α configuration increases the latency to foot withdrawal in the hot plate test in mice (15,27). To assess the possibility that progestins facilitate analgesia by acting directly on spinal GABAergic receptors we evaluated the capacity of PROG and three of its ring A-reduced metabolites to induce analgesia when administered IT either alone or in combination with a subanalgesic dose of MUSC.

METHODS

Animals

Female Wistar rats (250–300 g body weight) raised in our colony were used. They were kept in collective cages (six animals per cage) in a controlled light (14 h light : 10 h dark) and temperature (23 ± 2°C) environment. Purina rat chow and water were available ad lib.

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Surgery

Subjects (Ss) were bilaterally ovariectomized under ether anesthesia. This procedure prevents endogenous fluctuations of ovarian steroids to influence the interaction of GABA agonists with the $GABA_A$ receptor (18,20,21). Two weeks after ovariectomy, under pentobarbital anesthesia (35 mg/kg, IP), a catheter (Clay-Adams PE-10 polyethylene tubing, Fisher Chemical, Springfield, NJ) was implanted chronically into the subarachnoid IT space through an incision made in the atlanto-occipital membrane (30). The tip of the catheter (7.5 cm insertion length) reached the lumbo-sacral level of the spinal cord. Only Ss showing no motor disturbances after surgery were used in the experiment. At least one week of recovery was allowed before testing. After completion of the study, Ss were anesthetized with ether, perfused through the heart with 0.9% saline/10% formalin, and the spinal cord was dissected to verify correct placement of the catheter. Only Ss with catheters placed correctly were included in the analysis.

Behavioral Testing

The nociceptive threshold (NT) of all Ss was determined in the vocalization threshold to tail shock (VTTS) and tail-flick latency (TFL) tests before drug infusion. VTTS was determined by placing rats inside a Plexiglas restrainer and taping two stainless steel electrodes to the tail after applying conductive gel. Electric shocks (100-ms trains of 60-Hz square pulses; duration = 0.1 ms) were delivered through a constant current shock generator (Nuclear Chicago, Des Plaines, IL) via tail electrodes. The amperage was increased in 100-μA steps until vocalization was elicited (upper shock level) and then decreased stepwise until vocalization ceased (lower shock level). This procedure was repeated three times. Upper shock levels were averaged to provide an estimate of VTTS. Only rats vocalizing between 300 and 800 μA were used. This selection procedure, which excluded about 20% of the initially tested Ss, was used to discard 1) "irritable rats," which vocalized to neutral tactile stimulation, and 2) Ss which would require intense, potentially damaging, shocks to vocalize and would therefore require a longer testing time inside the Plexiglas restrainer. TFL was determined with an IITC (Woodland Hills, CA) Model 333 Analgesimeter at 90% beam intensity. TFL was measured automatically by activation of a photocell upon tail withdrawal. Each test score was the average of three trials. A cutoff time of 15 s of exposure to the radiant heat was used to avoid tissue damage.

Infusion Procedure and Treatment Groups

Two weeks after implantation of the catheter, Ss were injected IT with either absolute ethanol (vehicle), muscimol hydrobromide (MUSC), progestin, or progestin plus MUSC. Rats were used twice in a balanced design in which they were allotted at random to any 2 of 30 groups. Two weeks elapsed between the two tests. Progestins at the following doses were used: 4-pregnen-3,20-dione (progesterone: 0.25, 2.5, 25, and 250 μg), 3α-hydroxy-5α-pregnan-20-one (5α,3α-pregnanolone: 0.25, 2.5, 25, and 250 μg), 3α-hydroxy-5β-pregnan-20-one (5β,3α-pregnanolone: 2.5, 25, and 250 μg), and 3β-hydroxy-5β-pregnan-20-one (5β,3β-pregnanolone: 2.5, 25, and 250 μg). Progestins were tested alone and in combination with MUSC. The dose of MUSC (1 μg) was selected from previous studies showing that it does not induce analgesia per se (25). Steroids were obtained from Sigma Chemical Co. (St. Louis), and muscimol hydrobromide was purchased from Research Biochemicals Inc. (Natick, MA). All drugs were dissolved in 5 μl ethanol. (See Tables 1 and 2 for number of Ss in each group.)

After determining the NT, rats were injected IT with 5 μl ethanol, containing the drug, plus an additional 7 μl of saline for flushing. During IT injections (lasting around 1 min), Ss were kept in a Stoelting animal holder. Immediately after in-

TABLE 1

EFFECT OF INTRATHECAL ADMINISTRATION OF ETHANOL AND VARIOUS PROGESTINS ON THE VOCALIZATION THRESHOLD TO TAIL-SCHOCK (MEANS ± SE; MICROAMPERES)

Treatment	N	Preinjection	Minutes Postinjection			
			3	10	20	30
Ethanol	12	594 ± 28	525 ± 51	450 ± 62	483 ± 46	474 ± 55
Progesterone						
0.25 μg	10	606 ± 47	536 ± 47	493 ± 37	520 ± 34	516 ± 22
2.5 μg	7	538 ± 74	500 ± 63	414 ± 43	452 ± 37	461 ± 81
25 μg	6	522 ± 70	494 ± 90	466 ± 79	410 ± 59	422 ± 60
250 μg	4	599 ± 61	608 ± 39	566 ± 26	500 ± 100	566 ± 31
5α,3α-Pregnanolone						
2.5 μg	9	622 ± 40	585 ± 35	533 ± 50	522 ± 36	548 ± 43
25μg	7	661 ± 23	652 ± 62	647 ± 58	590 ± 71	609 ± 64
25 0 μg	4	541 ± 43	533 ± 26	475 ± 43	491 ± 49	533 ± 59
5β,3α-Pregnanolone						
2.5 μg	9	537 ± 52	392 ± 51	433 ± 29	481 ± 43	485 ± 50
25 μg	7	538 ± 43	466 ± 66	490 ± 53	590 ± 64	514 ± 63
250 μg	5	633 ± 34	579 ± 146	380 ± 95	333 ± 71	446 ± 75
5β,3β-Pregnanolone						
2.5 μg	9	503 ± 36	470 ± 43	503 ± 43	510 ± 42	518 ± 37
25 μg	9	611 ± 49	670 ± 142	648 ± 111	655 ± 116	640 ± 121
250 μg	10	480 ± 29	479 ± 32	450 ± 30	473 ± 36	516 ± 39

TABLE 2

EFFECT OF INTRATHECAL ADMINISTRATION OF VARIOUS PROGESTINS ON THE TAIL-FLICK LATENCY (MEANS ± SE; SECONDS)

Treatment	N	Preinjection	Minutes Postinjection			
			3	10	20	30
Progesterone						
0.25 μg	10	8 ± 0.5	8 ± 0.6	8 ± 0.6	8 ± 0.6	8 ± 0.5
2.5 μg	7	8 ± 0.8	9 ± 1	7 ± 0.9	8 ± 1	7 ± 0.6
25 μg	6	7 ± 0.4	7 ± 0.8	7 ± 0.4	7 ± 0.6	7 ± 0.6
250 μg	4	5 ± 0.5	5 ± 0.7	5 ± 0.6	5 ± 0.7	5 ± 0.9
5α,3α-Pregnanolone						
2.5 μg	9	7 ± 0.5	7 ± 0.6	7 ± 0.6	7 ± 0.7	7 ± 0.6
25 μg	7	8 ± 0.5	8 ± 0.4	8 ± 0.4	8 ± 0.8	8 ± 0.6
250 μg	4	8 ± 0.3	9 ± 0.4	8 ± 0.3	8 ± 0.3	8 ± 0.3
5β,3α-Pregnanolone						
2.5 μg	9	7 ± 0.7	7 ± 0.5	7 ± 0.6	7 ± 0.5	7 ± 0.8
25 μg	7	6 ± 0.7	6 ± 0.6	5 ± 0.5	6 ± 0.7	5 ± 0.5
250 μg	5	6 ± 2	6 ± 0.4	7 ± 0.2	5 ± 0.5	6 ± 0.2
5β,3β-Pregnanolone						
2.5 μg	9	9 ± 0.3	9 ± 0.2	9 ± 0.2	9 ± 0.4	10 ± 0.2
25 μg	9	9 ± 0.2	9 ± 0.2	9 ± 0.4	10 ± 0.2	10 ± 0.2
250 μg	10	8 ± 0.2	8 ± 0.5	8 ± 0.5	8 ± 0.5	8 ± 0.5

jection, Ss were placed in a Plexiglas cage and observed for behavioral or motor alterations. Observers ignored which drug had been injected. To determine the time course of drug-induced effects VTTS and TFL tests were performed at 3, 10, 20, and 30 min postinjections.

Statistical Analysis

The effect of each progestin alone on NT was analyzed in comparison with the vehicle using an ANOVA for repeated measures on one factor (3). The effect of progestins combined with MUSC on NT was analyzed in comparison with both the vehicle and the MUSC groups using the same ANOVA described above. This analysis allows comparisons of the differences in the overall performance of Ss in all groups (i.e., several doses of progestins and the vehicle). It also evaluates changes in the performance of Ss across experimental sessions (i.e., time intervals following drug administration). When appropriate, this procedure was followed by a Scheffé test (3). This test allowed us to determine which specific means differed significantly from each other. The effects provoked by the IT administration of ethanol alone were assessed by comparing preinjection values with those observed at 3, 10, 20, and 30 min postinjection. A Wilcoxon test was used for these comparisons.

RESULTS

Effect of Ethanol and MUSC on NT

The i.t. administration of ethanol plus saline did not increase the NT in either the VTTS or the TFL. On the contrary, values following ethanol administration were consistently lower than preinjection values in the VTTS test at 10 ($p < 0.025$), 20 ($p < 0.025$), and 30 ($p < 0.01$) min postinjection (Table 1 and Fig. 1). By contrast, IT administration of ethanol did not modify the TFL (Table 3). The administration of 1 μg MUSC also failed to induce significant analgesia in both the VTTS (Fig. 1) and the TFL (Table 3) tests, values not differing significantly from those of the vehicle.

Effect of Progestins Given Alone on NT

Table 1 summarizes the effects on the VTTS provoked by the various progestins. ANOVA failed to reveal any significant effect of these progestins at the level of $p < 0.05$. Analysis of the table reveals that in most cases values tended to decline following IT injection of progestins. A similar trend, however, was observed with the vehicle alone. Table 2 shows that none of the progestins given alone modified the TFL at any of the doses tested.

Effect of Progestins Combined With Muscimol on NT

Figure 1 shows that the combined administration of MUSC and PROG produced significant analgesia in the VTTS test. All groups that combined PROG plus MUSC showed overall values significantly higher than those obtained with the vehicle. However, only the 2.5-μg dose was significantly different from the overall values produced by 1 μg MUSC. As can be seen in Fig. 1, the rise in VTTS occurred almost immediately (3 min) and tended to remain high throughout the duration of the experiment. All doses of 5α,3α-pregnanolone combined with MUSC induced significant analgesia in comparison with ethanol, and the two largest doses (25 and 250 μg) also in comparison with MUSC (Fig. 1). As in the case of PROG, a clear rise over preinjection values was already noted at 3 min, though peak values occurred between 10 and 20 min, thereafter slowly declining. The combined administration of 5β,3α-pregnanolone plus MUSC significantly increased the VTTS, against both the vehicle (ethanol) and MUSC, only at the 25-μg dose (Fig. 1). A sharp rise in the VTTS was already apparent at 3 min, and the values continued rising until 20 min. By contrast, with the dose of 250 μg, only a transient increase in the VTTS was noted at 3 min, with values sharply

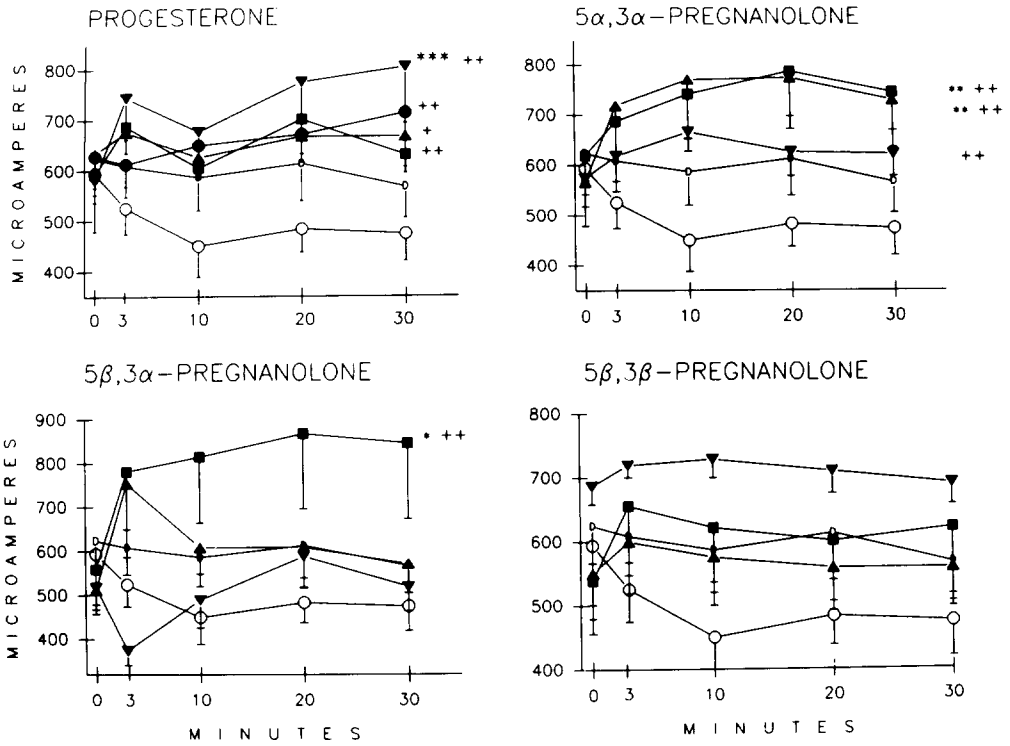

FIG. 1. Effect on the VTTS test of the IT administration of ethanol (n = 12, ○), 1 μg muscimol (n = 8, open half-circles), or one of the following doses of progestins combined with 1 μg muscimol: progesterone, 0.25 μg (n = 8, ●), 2.5 μg (n = 10, ▼), 25 μg (n = 8, ■), 250 μg (n = 4, ▲); 5α,3α-pregnanolone, 2.5 μg (n = 9, ▼), 25 μg (n = 8, ■), 250 μg (n = 9, ▲); 5β,3α-pregnanolone, 2.5 μg (n = 9, ▼), 25 μg (n = 9, ■), 250 μg (n = 8, ▲); 5β,3β-pregnanolone, 2.5 μg (n = 7, ▼), 25 μg (n = 6, ■), 250 μg (n = 4, ▲). Data show means minus standard errors obtained at different time intervals after IT injections. *p < 0.05; **p < 0.025; ***p < 0.01 vs. muscimol; +p < 0.05; + +p < 0.005 vs. ethanol.

declining thereafter. The combined administration of 5β,3β-pregnanolone plus MUSC did not significantly increase the VTTS at any of the doses tested (Fig. 1).

No significant effects on the TFL were noted after the IT administration of any of the combinations of progestins plus MUSC (see Table 3).

DISCUSSION

The present results suggest that some progestins can interact with spinal $GABA_A$ receptors to enhance the inhibitory action exerted by $GABA_A$ agonists on spinal neurons involved in the transmission of pain. Surprisingly, progestins given alone did not produce significant analgesia. This failure may be due to a low discharge rate of the spinal GABAergic system under normal conditions, thereby releasing very low amounts of GABA to synergize with the injected progestin. It is possible that the reported analgesic effect of IT injections of 5α,3α-pregnanolone in a mechanical visceral pain model (28) was due to the fact that the noxious stimulus used in such study (balloon distension of the duodenum) provokes a more intense and prolonged activation of the spinal GABAergic system than that produced by our brief, threshold shocks to the tail.

Our data is consistent with numerous in vitro results showing the capacity of some pregnane derivatives (progestins and corticoids) to increase the effect of GABA agonists on the $GABA_A$ receptor (11,12,18,20). This agreement between in vitro and in vivo studies is, however, not trivial, since in some cases such relationship does not occur. Thus, although data from most biochemical studies clearly demonstrate that ethanol potentiates $GABA_A$ receptor function, electrophysiological studies are strikingly inconsistent in this regard [for review see (17)]. Moreover, analysis of neuronal activity shows that $GABA_A$ receptors are modulated by ethanol in some CNS regions but not in others. Therefore, specific studies should assess the action of a drug potentially modulating GABAergic activity in circumscribed neural regions possessing GABAergic innervation. In agreement with biochemical data (11,12, 18,20), ring A-reduced progestins showing a 5α or 5β configuration combined with the 3α-hydroxy configuration clearly potentiated the effect of MUSC to induce analgesia in the VTTS. Conversely, 5β,3β-pregnanolone provoked only a modest, not statistically significant effect. Nonetheless, the stringency of our statistical analysis (comparisons between groups irregardless of preinjection values) may have precluded the detection of small but meaningful effects. On the other hand, PROG, a delta-4,3-keto pregnane, also significantly facilitated analgesia when combined with MUSC, even at a lower dose than its ring A-reduced metabolites. This last result was somewhat surprising, since in most biochemical studies 3α-hydroxy pregnanes were more potent than PROG as regards interacting with the $GABA_A$ receptor (11,12,18,20). However, it is possible that PROG, through only two enzy-

TABLE 3

EFFECT OF INTRATHECAL ADMINISTRATION OF ETHANOL, MUSCIMOL (MUSC), AND VARIOUS PROGESTINS PLUS MUSCIMOL ON THE TAIL-FLICK LATENCY (MEANS ± SE; SECONDS)

Treatment	N	Preinjection	Minutes Postinjection			
			3	10	20	30
Ethanol	5	5 ± 0.4	5 ± 0.4	5 ± 0.7	5 ± 0.4	5 ± 0.4
Muscimol	5	6 ± 0.4	6 ± 0.5	5 ± 0.2	5 ± 0.2	5 ± 0.2
Progesterone + MUSC						
0.25 μg	8	9 ± 0.5	9 ± 0.4	9 ± 0.5	9 ± 0.3	9 ± 0.5
2.5 μg	10	8 ± 0.6	8 ± 0.9	8 ± 1.0	7 ± 1.1	7 ± 0.9
25 μg	8	5 ± 0.2	5 ± 0.4	6 ± 0.5	5 ± 0.5	4 ± 0.3
250 μg	4	8 ± 1.6	6 ± 0.4	5 ± 0.5	5 ± 0.8	5 ± 0.9
5α,3α-Pregnanolone +MUSC						
2.5 μg	9	9 ± 0.2	10 ± 0.1	10 ± 0.2	10 ± 0.2	9 ± 0.2
25 μg	8	8 ± 0.3	8 ± 0.3	8 ± 0.2	8 ± 0.5	9 ± 0.3
250 μg	9	9 ± 0.4	9 ± 0.2	8 ± 0.7	9 ± 0.4	9 ± 0.3
5β,3α-Pregnanolone +MUSC						
2.5 μg	9	6 ± 0.3	6 ± 0.5	6 ± 0.4	6 ± 0.4	7 ± 0.5
25 μg	9	8 ± 0.7	8 ± 0.4	9 ± 0.6	9 ± 0.5	9 ± 0.6
250 μg	8	8 ± 0.5	7 ± 0.5	7 ± 0.5	8 ± 0.5	8 ± 0.7
5β,3β-Pregnanolone +MUSC						
2.5 μg	7	9 ± 0.5	8 ± 0.7	9 ± 0.7	9 ± 0.7	9 ± 0.6
25 μg	6	8 ± 0.5	7 ± 0.6	7 ± 0.5	8 ± 0.5	8 ± 0.4
250 μg	4	8 ± 1.0	8 ± 1.0	8 ± 1.1	8 ± 1.1	8 ± 1.1

matic steps (5α- and 3α-reduction), was metabolized in the spinal cord to its more potent derivatives (14). This conversion could result in a more adequate local distribution of the pregnanolone molecules around the $GABA_A$ receptor than that achieved by the direct perispinal administration of the 3α-reduced pregnanolones.

Besides acting on the $GABA_A$ receptor, progestins can exert several actions on neurons potentially modifying pain perception. Thus, PROG can antagonize the action of glycine on its strychnine-sensitive receptor [gly 1 (29)], an effect that theoretically could decrease nociceptive thresholds, as occurs when gly 1 receptors are blocked by strychnine (2). Moreover, antagonistic effects on opiate receptors have been reported for 3α-hydroxy pregnanolones in mice (8). Furthermore, large doses of ring A-reduced progestins can inhibit the release of neurotransmitters [e.g., noradrenaline; (16)] known to inhibit the transmission of nociceptive impulses at the spinal cord (5,9,23). Therefore, it was not surprising that no clear linear dose-response relationships were noted following progestin-MUSC combinations, since it is possible that at certain doses progestins exerted effects on other systems which were opposite to those induced through the enhancement of $GABA_A$ receptor activity.

Vocalization in response to noxious stimulation of the tail is an affective, motivational component of pain (13). On the other hand, the TFL test is a reflexive pain test which assesses the sensory, discriminative dimension of pain (7). These two responses associated to noxious stimulation involve the activation of different neural systems. Our results showing that progestins combined with MUSC increased the VTTS without affecting the TFL would suggest that spinal GABAergic neurons mainly modulate ascending pain messages, but not those mediating the tail flick reflex to noxious thermal stimulation. However, IT administration of some $GABA_A$ agonists like THIP and MUSC have been reported to increase the TFL in both mice and rats (10,25), thus suggesting the operation of $GABA_A$ receptors on neurons mediating this response. Therefore, the differential sensitivity of VTTS versus TFL to the combination of progestins plus MUSC may reflect the operation of other factors like bioavailabilty (i.e., a preferential access of the agents used to one population of neurons) rather than an absence of $GABA_A$ receptors in the neural system mediating TFL. Alternatively, this differential sensitivity may suggest that the interaction of progestins with the $GABA_A$ receptor is specific for those neural systems modulating ascending but not reflexive responses, despite the involvement of $GABA_A$ receptors in both. Differential responses to analgesic drugs, including $GABA_A$ agonists, have been frequently reported in the literature depending on the nociception tests used. Thus, IT THIP administration in mice increases the hot-plate but not the tail-flick latencies, while, surprisingly, in this same preparation MUSC increases tail-flick but not hot-plate latencies (10).

In summary, our results demonstrate that some progestins can enhance the action of GABAergic drugs at the $GABA_A$ postsynaptic receptors in the spinal cord. These data may have important implications in the perception of pain under conditions where progestin plasma levels are high (e.g., across pregnancy or during the intake of medications containing potent synthetic progestins). Indeed, a decreased sensitivity to noxious stimulation during pregnancy has been reported in women (6). To our knowledge, no study has explored the pain

thresholds of women taking synthetic progestin medications or the actions of ring A-reduced metabolites of synthetic progestins on the $GABA_A$ receptor in vitro. Future studies should assess these important issues in view of the common use of synthetic progestins for birth control and for treating acne and menstrual cycle disorders.

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