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Interaction of GABA and Serotonin in the Anxiolytic Action of Diazepam and Serotonergic Anxiolytics

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LÓPEZ-RUBALCAVA, C., A. SALDÍVAR AND A. FERNÁNDEZ-GUASTI. *Interaction of GABA and serotonin in the anxiolytic action of diazepam and serotonergic anxiolytics.* PHARMACOL BIOCHEM BEHAV 43(2) 433–440, 1992.– The general purpose of the present study was to analyze the possible interactions between the GABA–benzodiazepine and the serotonergic (5-HT) systems in the anxiolytic action of diazepam and the 5-HT_{1A} agonists, ipsapirone, indorenate, and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). The effect of the benzodiazepine receptor antagonist, flumazenil (10.0 mg/kg), on the anxiolytic action of ipsapirone (5.0 mg/kg), indorenate (5.0 mg/kg), and 8-OH-DPAT (0.125 mg/kg) was examined on the avoidance exploratory behavior paradigm in mice. The effect of the 5-HT_1 blockers, methiotepin (0.31 mg/kg), pindolol (3.1 mg/kg), and alprenolol (5.0 mg/kg), on the anxiolytic action of diazepam (0.5 mg/kg) was also studied. In the last part of this work, the putative potentiation between diazepam (0.25 mg/kg) and each of the serotonergic anxiolytics was investigated. The antianxiety effect of diazepam, ipsapirone, indorenate, and 8-OH-DPAT was prevented by flumazenil. The serotonergic/β-blocker, alprenolol, partially antagonized the diazepam effect. Finally, a potentiation of suboptimal doses of diazepam and ipsapirone, but not with indorenate or 8-OH-DPAT, was observed. The findings suggest an interaction between both systems on the anxiolytic action of diazepam and the 5-HT_{1A} agonists.

5-HT_{1A}- anxiolytics Diazepam Flumazenil 5-HT_1/β-blockers GABA–5-HT interactions Anxiety

TWO main neurotransmitter pathways have been proposed to participate in the physiopharmacological regulation of anxiety: the benzodiazepine–GABA and the serotonergic systems (25,26,32).

At present, it is widely accepted that the mechanism through which the already classic anxiolytics, benzodiazepines, produce their antianxiety action involves the interaction of the benzodiazepine receptor with the $GABA_A$ site (22,47). Recently, serotonin (5-HT) has been implicated in the regulation of anxiety; however, its precise function remains unclear. In general terms, it seems that the increase of endogenous 5-HT produces proconflict effects, while a reduction or a blockade of the 5-HT transmission results in anxiolytic actions [for review, see (25,26)]. Conversely, it is generally agreed that administration of agonists to the 5-HT_{1A} receptor produces anxiolytic effects in several anxiety models [for review, see (10)]. These new anxiolytic drugs are structurally unrelated to benzodiazepines (39), and there is no evidence for a direct interaction of the serotonergic anxiolytics with the benzodiazepine receptor (9,23,46).

Because benzodiazepines inhibit the firing of serotonergic neurons in the dorsal raphé nucleus (40,50) and decrease the 5-HT turnover (7,29), it was suggested that benzodiazepines might produce their anxiolytic effects by acting upon serotonergic neurons. However, Thiébot et al. (48) found that even after lesioning the serotonergic neurons the benzodiazepines were able to produce their anxiolytic actions.

Although a direct interaction of benzodiazepines with the serotonergic receptors seems unlikely, several anatomical (5,21,52), neurophysiological (45), and biochemical (33,34) data reveal important interactions between the GABA–benzodiazepinic and serotonergic systems. These interactions have been described in brain areas closely related with the regulation of anxiety (18,28), and some of them involve the serotonin receptor subtype, 5-HT_{1A}, most likely implied in the action of the serotonergic anxiolytics (20,37).

Therefore, the general purpose of the present work was to analyze the possible relationships between these two neurotransmitter systems in the regulation of anxiety. Three specific objectives were included as follows: a) the putative antago-

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nism of the anxiolytic effect of the serotonergic compounds, ipsapirone, indorenate, and 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT) by the selective benzodiazepine antagonist, flumazenil; b) the possible blockade of the diazepam anxiolytic effect by the serotonin antagonists, methiotepin, pindolol, and alprenolol; and c) the putative potentiation between diazepam and each of the serotonergic anxiolytics. Additional series of control experiments studying the motor activity were included.

METHOD

Animals

Male Swiss-Webster mice (20–30 g) were used in these experiments. All mice were housed in groups of five per cage with food and water freely available. Animals were maintained in a room under controlled and inverted light-dark cycle (12 L : 12 D, lights on at 1000 h) conditions.

Anxiety Test

The avoidance exploratory behavior shown by mice and described by Crawley and Goodwin in 1980 (8) and Blumstein and Crawley in 1983 (3) was used to test anxiety. This method consisted of placing the animal in a propylene cage (44 × 21 × 21 cm) divided in two compartments. One compartment (one third of the cage) was completely blackened on all surfaces; the other compartment (two thirds of the cage) was highly illuminated by a fluorescent lamp. An opening of 5 × 13 cm separated the dark from the bright compartment. At the beginning of the test, the animal was placed in the bright compartment. The number of transitions from one compartment to the other were recorded in a 10-min session. In these series of experiments, a balanced Latin square design was used. Within each series, animals were randomly divided into four groups, each group receiving different treatments in each session in such a way that every animal received all treatments. A 3-day interval was left between the tests. The data were statistically compared using the Friedman two-way analysis of variance (ANOVA) followed by the Wilcoxon matched-pairs signed-ranks test (43).

Activity Test

The motor activity was recorded in a box measuring 43 × 36 × 19 cm that was placed over a sensitive plaque (48 × 40 cm) of an activity meter (Stoelting Co., Chicago, IL) connected to a counter (Stoelting Co.) The animal was placed in the cage and the number of counts were recorded over a 10-min period. Between each test, the cage was carefully cleaned. Data are expressed as number of counts per minute. Because animals could not be tested more than once, in these series of experiments an independent group design was used. The data were statistically analyzed by help of the Mann-Whitney *U*-test (43). All combined treatments used in the exploratory behavior experiments were studied on the motor activity test (see Table 1).

Drugs

The drugs used in this study were: indorenate (CINVESTAV-Miles, México City, México), ipsapirone (Miles Pharmaceutical Division, West Haven, CT), 8-OH-DPAT (Research Biochemicals Inc., Natick MA), pindolol (Sandoz, Basel, Switzerland), alprenolol (Hässle AB, Mòndal, Sweden), methiotepin (Hoffman-La Roche, Basel, Switzerland), diazepam (Hoffman-La Roche, México City, México), and flumazenil [(Ro 15-1788) Hoffman-La Roche, México City, México]. All drugs were injected IP in volumes of 5 ml/kg. Diazepam was dissolved in propylene glycol 40%. Flumazenil was dissolved in distilled water with a drop of Tween-80. All other drugs were dissolved in physiological saline.

Experiment 1: Effect of the Benzodiazepine Antagonist Flumazenil on the Anxiolytic Action of Serotonergic Agonists

In these series of experiments, the drugs were injected as follows: a) ipsapirone (5.0 mg/kg) + flumazenil (10.0 mg/kg), −30 min; b) indorenate (5.0 mg/kg) + flumazenil (10.0 mg/kg), −90 min; and c) 8-OH-DPAT (0.125 mg/kg) + flumazenil (10.0 mg/kg), −30 min. The proper control groups for each treatment were included. The doses and the latencies for each of the serotonergic anxiolytics were established according to previous data (13).

Experiment 2: Effect of Various 5-HT/β-Antagonist on the Anxiolytic Action of Diazepam

In this experiment, the following treatments were included: a) diazepam (0.5 mg/kg) + flumazenil (10.0 mg/kg), −30 min; b) diazepam (0.5 mg/kg) + pindolol (3.1 mg/kg), −30 min; c) diazepam (0.5 mg/kg) + alprenolol (5.0 mg/kg), −30 min; and d) diazepam (0.5 mg/kg) + methiotepin (0.31 mg/kg), −30 min. As for the previous series, the proper control groups were included for each treatment.

Experiment 3: Possible Potentiation of the Anxiolytic Action of Diazepam and Serotonergic Anxiolytics

The groups included in these experimental series were: a) diazepam (0.25 mg/kg) + ipsapirone (2.5 mg/kg), −30 min; b) diazepam (0.25 mg/kg) + indorenate (2.5 mg/kg), −90 min; and c) diazepam (0.25 mg/kg) + 8-OH-DPAT (0.0625 mg/kg), −30 min. The low subthreshold doses of the compounds used in these treatments were selected according to previously established dose-response curves (13).

RESULTS

Experiment 1: Effect of the Benzodiazepine Antagonist Flumazenil on the Anxiolytic Action of Serotonergic Agonists

Figure 1 shows the results of this experiment. Clearly, administration of all three 5-HT_{1A} agonists, ipsapirone, indorenate, and 8-OH-DPAT, produced an increase on the exploratory behavior in mice evidenced as an increase in the number of transitions. In addition, the benzodiazepine antagonist, flumazenil (10.0 mg/kg), did not modify the number of transitions per se as compared with its respective control group. Interestingly, flumazenil completely blocked the anxiolytic action of ipsapirone and partially antagonized the effect of indorenate and 8-OH-DPAT.

Experiment 2: Effect of Various 5-HT/β-Antagonists on the Anxiolytic Action of Diazepam

Figure 2 shows the effect of the 5-HT antagonists, pindolol, alprenolol, and methiotepin, and that of the benzodiazepine antagonist, flumazenil, on the anxiolytic action of diazepam. Clearly, the administration of diazepam (0.5 mg/kg) produced an increase in the number of transitions in all four

TABLE 1

EFFECT OF VARIOUS DRUG COMBINATIONS ON MICE MOTOR ACTIVITY

Drug (dose, mg/kg)	*n*	Motor Activity (counts/min)
Saline control	10	30.40 ± 4.31
Diazepam (0.5) + flumazenil (10.0)	10	45.40 ± 5.71
Diazepam (0.5) + pindolol (3.1)	10	29.83 ± 3.68
Diazepam (0.5) + alprenolol (5.0)	10	33.50 ± 2.81
Diazepam (0.5) + methiotepin (0.31)	10	11.30 ± 3.83*
Ipsapirone (5.0) + flumazenil (10.0)	10	22.15 ± 2.86
Indorenate (5.0) + flumazenil (10.0)	10	23.48 ± 5.38
8-OH-DPAT (0.125) + flumazenil (10.0)	10	29.52 ± 3.03
Diazepam (0.25) + ipsapirone (2.5)	10	24.81 ± 3.85
Diazepam (0.25) + indorrenate (2.5)	10	33.75 ± 4.24
Diazepam (0.25) ± 8-OH-DPAT (0.0625)	10	22.84 ± 3.10

Results expressed as mean ± SE. Statistical comparisons made between the saline-treated group and experimental groups using the Mann-Whitney *U*-test.
*$p < 0.02$.

experiments. The 5-HT antagonists, pindolol (3.1 mg/kg), alprenolol (5.0 mg/kg), methiotepin (0.31 mg/kg), and the benzodiazepine antagonist, flumazenil (10.0 mg/kg), did not modify the exploratory behavior at the doses tested. Particularly interesting is the effect of the combined treatment of diazepam and the antagonists. Thus, a clear and complete antagonism of the diazepam anxiolytic action was observed after flumazenil and alprenolol injection. Methiotepin and pindolol, by contrast, only produce a partial antagonistic action.

Experiment 3: Possible Potentiation of the Anxiolytic Action of Diazepam and Serotonergic Anxiolytics

The results of this experiment are summarized in Fig. 3. From this figure, it is clear that only the combination of suboptimal doses of diazepam (0.25 mg/kg) plus ipsapirone (2.5 mg/kg) synergized to produce an increase in the number of transitions. The combined treatment of subthreshold doses of diazepam plus 8-OH-DPAT (0.0625 mg/kg) or diazepam plus indorenate (2.5 mg/kg) had no effect.

Table 1 shows the effect of the combination of diazepam plus the 5-HT agonists and antagonists and the combination of the 5-HT_{1A} agonists plus flumazenil on motor activity. As can be seen from Table 1, only the combination of diazepam (0.5 mg/kg) plus methiotepin (0.31 mg/kg) shows a statistically significant decrease on motor activity.

DISCUSSION

The main findings from the present study were:

1. The 5-HT_{1A} agonists, ipsapirone, indorenate, and 8-OH-DPAT, and the classic anxiolytic, diazepam, clearly produced an increase on exploratory behavior in mice, a response considered as an anxiolytic effect (8).
2. Administration of the benzodiazepine antagonist, flumazenil, prevented the anxiolytic action of ipsapirone, while it partially prevented the effect of indorenate and 8-OH-DPAT.
3. The combination of subthreshold doses of diazepam and ipsapirone (but not indorenate or 8-OH-DPAT) synergized in producing antianxiety effects.
4. The 5-HT/β-blocker, alprenolol (but not pindolol or methiotepin), effectively antagonized the anxiolytic actions of diazepam.
5. Only the combination of diazepam plus methiotepin affected motor activity.

In the present study, the benzodiazepine antagonist, flumazenil, did not produce any change in the exploratory behavior when administered alone as compared with the control group. However, it was able to prevent the anxiolytic effect of the serotonergic anxiolytics, ipsapirone, indorenate, and 8-OH-DPAT. Flumazenil has been reported to be a specific benzodiazepine receptor antagonist (4,24) without actions on the serotonergic receptor. Therefore, it appears that the benzodiazepine receptor complex is involved in the mediation of the anxiolytic effect of the 5-HT_{1A} agonists.

Recently, Söderpalm and Engel (44) reported that the GABA-benzodiazepine receptor complex could be involved in the anticonflict effect of parachlorophenylalanine (pCPA). These authors found that pCPA produced an anticonflict action in a modified Vogel's conflict model. This effect was completely counteracted by both flumazenil and bicuculline in doses not altering the behavior per se. In explaining their data, these authors included as one possibility that 5-HT normally exerts an inhibitory action on GABA release. Therefore, the abolishment of this inhibition (with pCPA pretreatment) would result in an increased GABAergic neurotransmission, which tentatively leads to an anxiolytic action. Following this line of thought, these series of results would suggest that stimulation of the 5-HT_{1A} receptor produces an inhibition of the serotonergic transmission that in turn causes an increased GABAergic transmission. It has been reported that the 5-HT_{1A} receptors are located both postsynaptically (on target cells of serotonergic neurons), and presynaptically on the cell bodies and/or dendrites of serotonergic neurons within the dorsal raphé nucleus (19,52). Because it has been demonstrated (2,53) that the stimulation of somatodendritic receptors results in a decreased serotonergic transmission, it seems possible that the

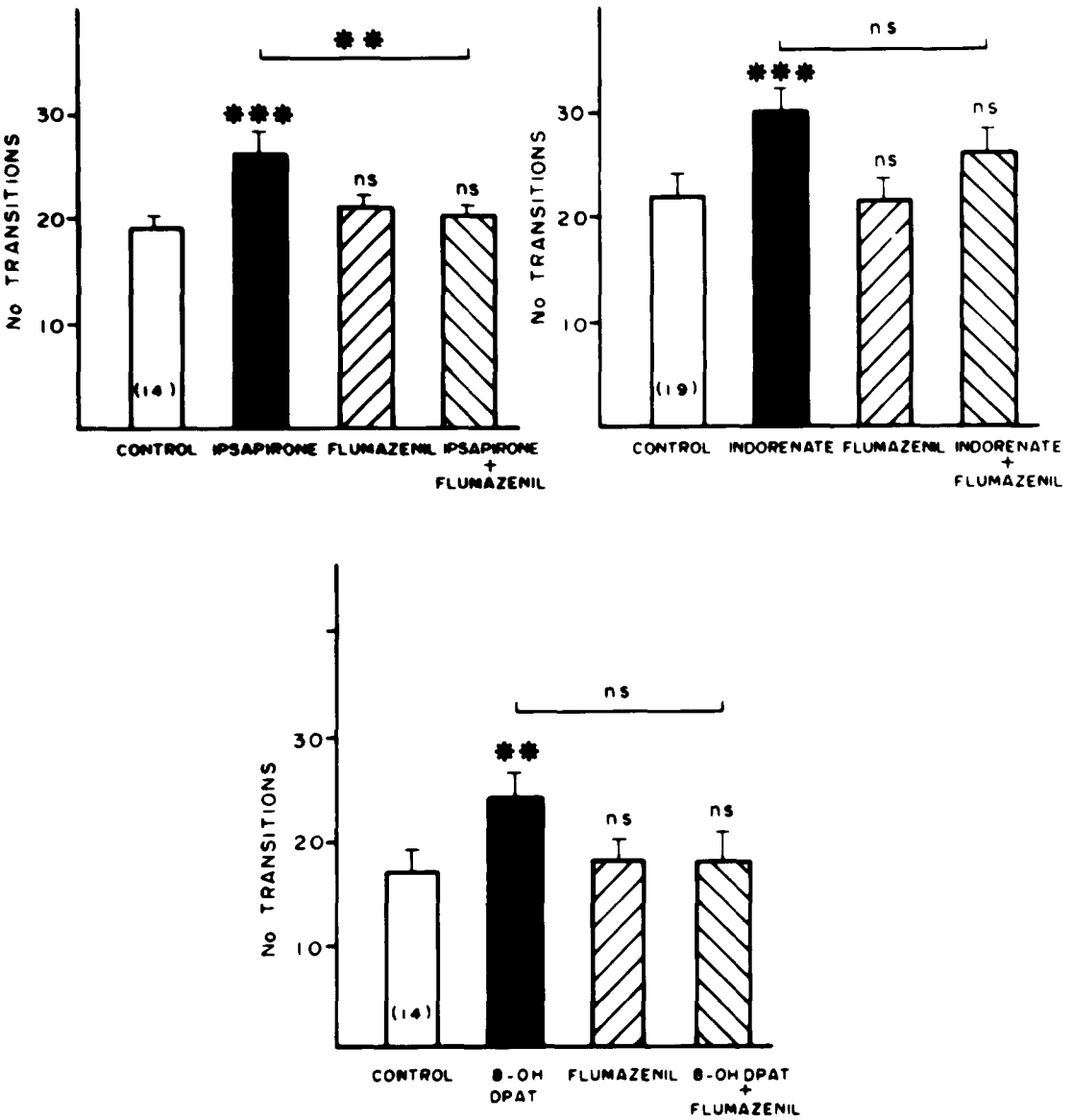

FIG. 1. Effect of the benzodiazepine antagonist flumazenil (10.0 mg/kg) on the anxiolytic action of ipsapirone (5.0 mg/kg), indorenate (5.0 mg/kg) and 8-OH-DPAT (0.125 mg/kg). Mean ± SE number of transitions. Statistical comparisons made between control and experimental groups (asterisks over columns) and between 5-HT_{1A} and 5-HT_{1A} plus flumazenil groups (brackets) by means of the Wilcoxon matched-pairs signed-rank test (**$p < 0.02$; ***$p < 0.01$).

serotonergic receptors involved in the anxiolytic effect of the 5-HT_{1A} agonists are located somatodendritically. Although this idea remains as a reasonable possibility (vide infra), a postsynaptic interaction (probably at the hippocampus) is also feasible.

Recently, we demonstrated that the integrity of the serotonergic somas is not essential for the action of the serotonergic anxiolytics, ipsapirone, indorenate, and buspirone (14). In this study, it was found that the 5-HT_{1A} agonists still produced their anxiolytic effect on the burying behavior test in animals in which the serotonergic neurons were lesioned with 5,7-dihydroxytryptamine (5,7-DHT, 150 μg) (14). According to these results, it was concluded that the 5-HT_{1A} agonists produced their anticonflict effect by the stimulation of postsynaptic neurons. These data are in accordance with those reported by Shimizu et al. (42). However, as previously suggested (12), we also found that the serotonergic somatodendritic receptors could be involved in the antianxiety effects of 8-OH-DPAT because in 5,7-DHT-lesioned rats 8-OH-DPAT does not produce an anxiolytic action (14). From these findings, it can be seen that the 5-HT_{1A} agonists exert their anxiolytic effects after the stimulation of either somatodendritic or postsynaptic receptors. However, the question regarding the site at which the GABA-benzodiazepine receptor complex interacts with the serotonergic system in producing an anxiolytic effect remains open.

The 5-HT_{1A} receptors appear particularly concentrated in limbic regions such as the hippocampus, septum, and amygdala (31,38,52). It is interesting to note that the hippocampus has been particularly implicated in the modulation of anxiety (18). For example, Kostowski et al. (27) reported that intrahippocampal injection of the 5-HT_{1A} receptor agonist, buspirone, produces an anxiolytic effect on the open-field and elevated plus-maze tests. In addition, it is important to note also that GABAergic neurons have been detected in the hippocampal area (30,36) and that Campbell et al. (6) found that local microinjection of diazepam into this brain region induced an anxiolytic effect on a conflict behavior paradigm. These data point out the hippocampus as an anatomic site where the serotonergic and GABAergic systems interact in the regulation of anxiety.

Another site where an interaction between the GABAergic and the 5-HT_{1A} systems might take place is the dorsal raphé nucleus. As aforementioned, there is evidence showing that the stimulation of somatodendritic 5-HT_{1A} receptors can produce antianxiety effects and that these receptors are located

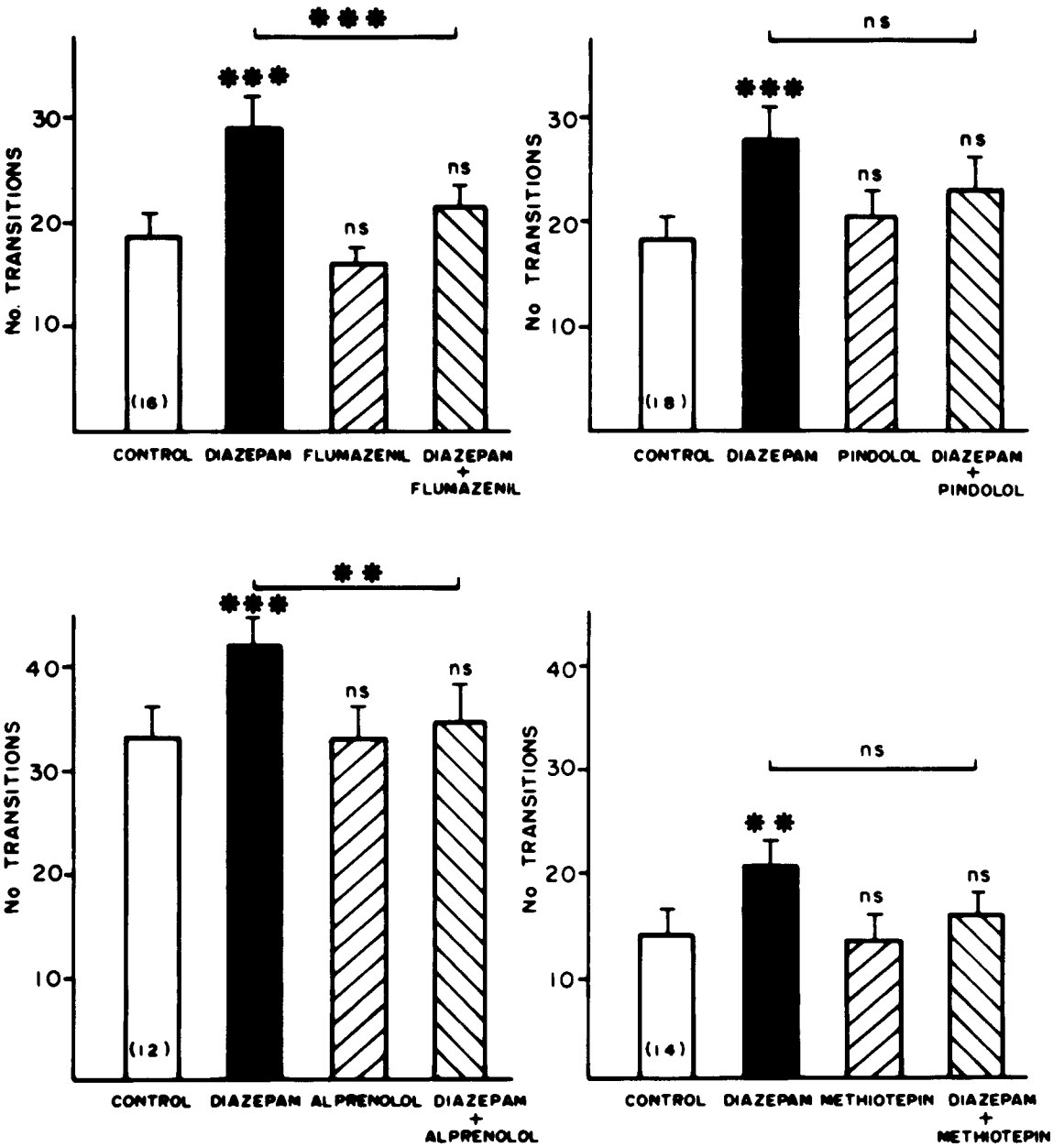

FIG. 2. Effect of the benzodiazepine antagonist, flumazenil (10.0 mg/kg), and the 5-HT/β-antagonists, pindolol (3.1 mg/kg), alprenolol (5.0 mg/kg), and methiotepin (0.31 mg/kg), on the anxiolytic action of diazepam (0.5 mg/kg). Mean ± SE number of transitions. Statistical comparisons made between control and experimental groups (asterisks over columns) and between diazepam and diazepam plus antagonists groups (brackets) by means of the Wilcoxon matched-pairs signed-rank test (**$p < 0.02$; ***$p < 0.01$).

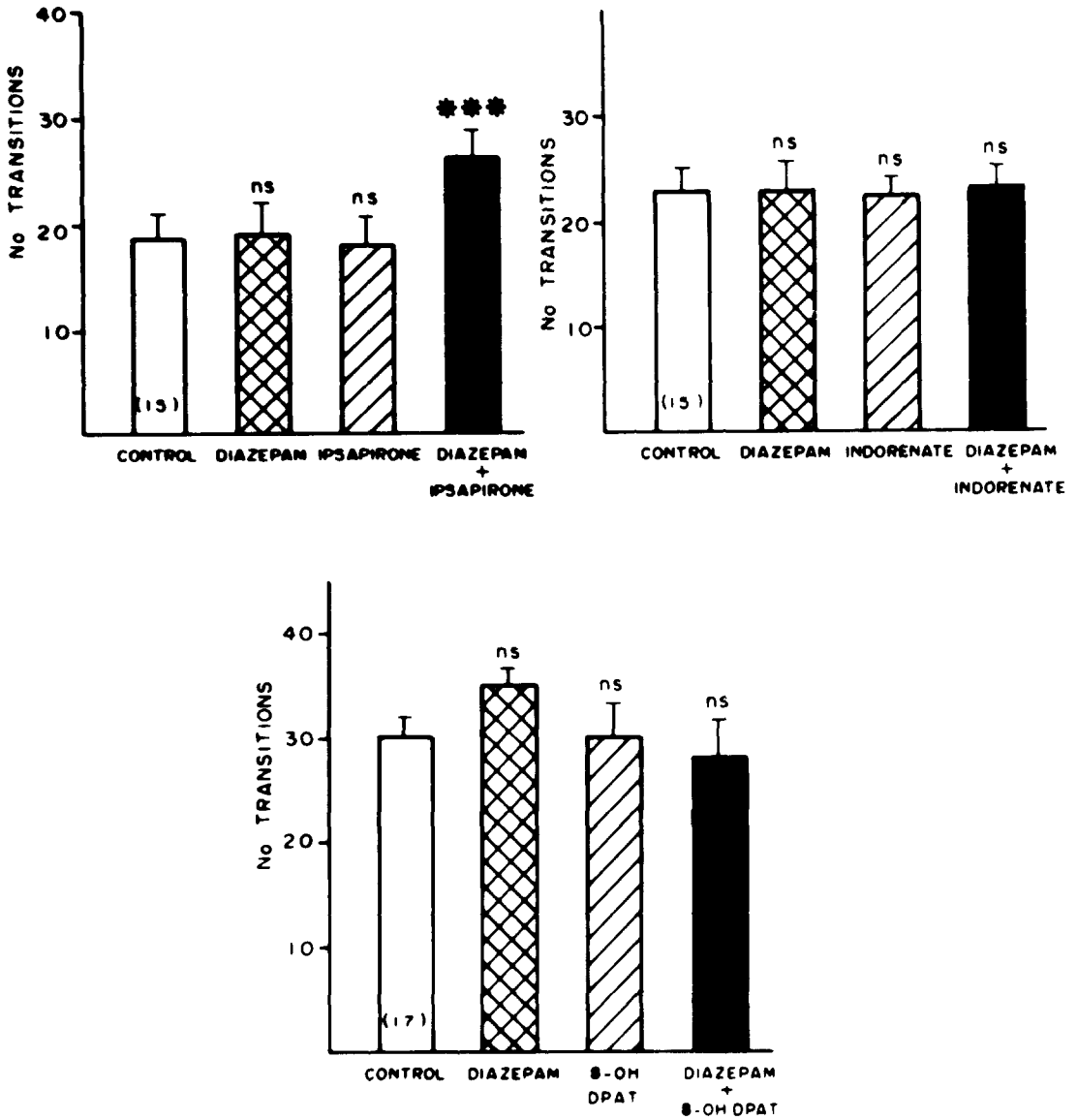

FIG. 3. Potentiation of subthreshold doses of diazepam (0.25 mg/kg) and the serotonergic anxiolytics, ipsapirone (2.5 mg/kg), indorenate (2.5 mg/kg), and 8-OH-DPAT (0.0625 mg/kg). Mean ± SE number of transitions. Statistical comparisons made between control (vehicle-treated) and experimental groups by means of the Wilcoxon matched-pairs signed-rank test (***$p < 0.01$).

on the dorsal raphé nucleus (51). Several authors have suggested that GABA acts as an inhibitor transmitter in the dorsal raphé nucleus (15,16). In addition, Thiébot et al. (49) reported that GABA and benzodiazepines applied to the doral raphé nucleus produced antianxiety effects while administration of β-carbolines to the same site produced anxiogenic actions. Furthermore, it has been demonstrated that benzodiazepines inhibit the firing of serotonergic neurons in the dorsal raphé nucleus (40,50) and decrease the 5-HT turnover (7). These results suggest an interaction of the GABAergic and serotonergic systems on the dorsal raphé nucleus. According to this idea, the inhibition of the serotonergic transmission produced by the stimulation of both the 5-HT_{1A} and GABA receptors in the somas of the serotonergic neurons would produce an anxiolytic effect and would also explain the present finding showing a potentiating action of the combined administration of diazepam and ipsapirone.

Other authors have reported a possible interaction of the 5-HT_{1A} agonist buspirone with the benzodiazepine receptor in other brain areas besides the raphé nucleus and the hippocampus. Thus, Garattini et al. in 1982 (17) reported that buspirone increases the binding of diazepam and GABA in the rat cerebellum. Following this idea, Oakley and Jones (35) found that buspirone dose dependently increased the amount of [^{3}H]flunitrazepam bound. These authors also showed that the threshold dose of buspirone that increased [^{3}H]flunitrazepam binding was the same required to produce an anxiolytic action on the Vogel's test. In addition, they reported that flumazenil was unable to prevent the anxiolytic action of buspirone. From these data, they concluded that the antianxiety effect of

this serotonergic anxiolytic does not depend upon the functional interaction of buspirone with benzodiazepine receptors and that the increase in [^{3}H]flunitrazepam binding might be related to the anxiolytic effect of buspirone mediated at some other site. Another evidence for interaction between buspirone and the GABAergic neurotransmission is that reported by Eison (11). This study showed that buspirone was able to reduce the convulsant threshold of the GABA antagonists, bicuculine and picrotoxin, when orally administered. It is worth mentioning that the exploratory behavior test in mice was not sensitive to the actions of buspirone (López-Rubalcava and Fernández-Guasti, unpublished data). Therefore, the possible interactions between buspirone and the GABA-benzodiazepine system cannot be explored in this particular test. Other experimental anxiety paradigms should be used to study such interaction.

From the present results, it can be hypothesized that the antianxiety effects seen in the present study are taking place by the stimulation of at least three different neurons connected linearly. The first neuron is serotonergic, having its origin on the dorsal raphé nucleus and providing 5-HT innervation to the hippocampus (1). This neuron, in addition to possessing the serotonergic somatodendritic receptors, would receive GABAergic information from an interneuron located in the raphé nucleus. The second neuron is of GABAergic nature, located in the hippocampus and probably innervated by the serotonergic fiber. Finally, there should be a third neuron that would be innervated by the GABAergic neuron. If this was the case, the 5-HT$_{1A}$ agonists could produce their anxiolytic effect by stimulating postsynaptic 5-HT$_{1A}$ receptors located in the hippocampal GABAergic neuron, while benzodiazepines would produce their anxiolytic action through postsynaptic stimulation of the third neuron. On the basis of this hypothesis, it would be explained why the benzodiazepine antagonist, flumazenil, counteracted the anxiolytic effect of the 5-HT$_{1A}$ agonists. However, it is important to state that, to our knowledge, there is no anatomic evidence for such an interaction in the hippocampus, and thus further studies should be done to confirm these suppositions.

In the present study, it was found that alprenolol was able to block the anxiolytic effect of diazepam. Alprenolol is a nonselective 5-HT$_{1A}$ receptor antagonist that also interacts with the β-adrenergic receptor but that, to our knowledge, does not interact with the benzodiazepine receptor. Other authors have reported interactions between 5-HT$_{1A}$/β-blockers and benzodiazepines. For example, Sepinwal et al. (41) found that a high dose of propanolol potentiated the actions of chlordiazepoxide on a drinking conflict paradigm; however, the mechanism of action for this effect was not discussed.

Because none of the treatments tested affected the mice motor activity (except for the combination of diazepam plus methiotepin, which decreased the activity), the results of the present work support the idea of an interaction between the benzodiazepine and serotonergic systems in the mediation of the anxiolytic effect of both diazepam and the 5-HT$_{1A}$ agonists.

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