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Effect of Flumazenil and Diazepam on Transient Actions in Defensive Burying Elicited by the Social Interaction Experience in Rats

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SALDIVAR-GONZALEZ, A., C., GOMEZ, I. MARTINEZ-LOMELI AND C. ARIAS. Effect of flumazenil and diazepam on transient actions in defensive burying elicited by the social interaction experience in rats. PHARMACOL BIOCHEM BEHAV **66**(2) 265–273, 2000.—In the present work, we studied the effects of benzodiazepine (BZ) receptor antagonist, flumazenil, and of the agonist, diazepam, on social interaction-induced transient changes in defensive burying (DB). Enhanced defensive burying was observed after 1.5 min of social interaction experience, while a longer social interaction experience, 15 min, inhibited the expression of burying behavior. Defensive burying and social interaction paradigms have been used for the screening of compounds with anxiolytic potential and, more extensively, to study the neurobiology of anxiety. To elucidate the participation of the BZ receptor on transient changes induced by intervals of social interaction experience, its receptor antagonist, flumazenil (2.5, 5, and 10 mg/kg) was intraperitoneally injected (IP). Flumzenil enhanced in a dose-dependent manner, the blocking effect of the saline IP injection on facilitated DB in 1.5-min social interaction-experienced subjects. In addition, flumazenil enlarged in a dose-dependent manner the blocking effect of saline IP on defensive burying levels in animals exposed to social interaction experience for 15 min. To analyze the presumed participation of the BZ receptor mediating enhanced burying behavior levels in subjects exposed to 1.5 min of social interaction, a suboptimal dose of diazepam (0.25 mg/kg) was administered. Diazepam enhanced the saline IP elicited defensive burying reduction. Results are discussed in terms of the suggested BZ receptor mediation on transient changes in defensive burying elicited by social interaction experience. © 2000 Elsevier Science Inc.

Anxiety Defensive burying Diazepam Flumazenil Social interaction

RECENTLY, our research group reported transient changes on defensive burying levels in rat, elicited by different lengths of experience in the social interaction paradigm (36), as well as the intraperitoneal saline injection (34). According to this evidence, rats experiencing 1.5 min of social interaction, showed facilitated burying behavior levels, an effect interpreted as increased emotional state, while animals spending a larger period (15 min) in the social interaction arena exhibited diminished burying values (36). We also observed that independent groups showed enhanced defensive burying when the interval between the IP saline injection and the anxiety test was carried out 1.5 min after manipulation and decreased when the emotional state was determined 3 min later (34). In addition, our group explored the temporal course of transient emotional changes elicited by social interaction experience (36) and IP saline injection (34). Defensive burying was minimized to basal levels 15 min after short social interaction, while animals that had remained for a long period in the arena showed an emotional state similar to that observed in control animals 30 min after finishing the social testing (36). The temporal course changes elicited by IP saline injection was explored as well (34). This experiment showed that 5 min after injection burying behavior decreased to baseline, remaining unchanged for as long as 30 min (34). These findings support the notion that emotional response in rats could be regulated according to the time elapsed after being delivered aversive stimuli (34,36). The differential expression on defensive burying, according to the time course analyzed, revealed

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a facilitated emotional response when burying behavior was studied soon after a stressful experience. On the other hand, there was an inhibition of burying levels when the interval between the experience and the emotional test was longer. The nature of the stressful experience seems to be relevant, because the inhibitory effect on defensive burying was observed early when the stimulus was the intraperitoneal saline injection, i.e., a nociceptive stimulus (34), and delayed in the case of social interaction test, a noninvasive procedure (36).

Changes in emotional responses after different experiences in birds and mammals have been described (8,13, 24,31,37,44,46). For instance, anxiogenic-like behavior was reported in rats after a brief training at the elevated plus-maze, an electric foot shock (8), and the exposure to cat odors (46). Recently, Fernándes and File (1996) and González and File (1997) have also described the effect of previous experience in animal paradigms developed for the screening of anxiolytic compounds, stating that experienced animals behave differentially when compared to naive subjects (9,18). Recently, González and co-workers published pharmacological evidence, suggesting that subtypes of benzodiazepine receptor differentially expressed in rat brain areas mediate different kinds of anxiety measured in two animal models (19).

Changes in the BZ receptor affinity in rat brains after being subjected to social interaction (31), cold swimming (27), and the plus-maze test (18) have been reported. On the other hand, antianxiety-like states expressed as inhibition of defensive burying in male rats have also been observed after ejaculation (13) or after enforced water drinking (35). These reports suggest that, under natural conditions, the animal's emotional responses could be modified, specifically after experiences that facilitate or block anxiety-like states (34–36).

The BZ subunit of the γ-aminobutyric acid ($GABA_A$) receptor complex has been proposed as a modulator of emotional responses in humans and animals (1–3,7,20,27,32). In addition, the BZ receptor antagonist, flumazenil (23) is now used as a specific antidote in case of suicidal or accidental benzodiazepine intoxication (4,5,21). Flumazenil has also been reported for its ability to revert anxiogenic action without intrinsic effects (28,29,34,45), as well as preventing stress-induced release of biogenic amines (31). Furthermore, diazepam has been widely evaluated for its ability to revert anxiety states in humans and to reduce anxiety-like responses in nonhuman experimental subjects (20). Thus, the aim of the present work was to study the putative role of the BZ receptor in the transient actions of defensive burying behavior elicited by previous social interaction experience in rats.

METHOD

Animals

Male Wistar rats (250–300 g) were used throughout the study. These were obtained from the Faculty of Medicine's animal house. The animals were handled following the Rules of Research in Health Matters (Ministry of Health, México) with the approval of the local Animal Care Committee. Animals were kept in an inverted light–dark cycle room (0800–2000h) with access to food and water ad lib and were housed six per cage (55 × 35 × 20 cm). Rats were placed in individual home cages 72 h before the experiments.

Anxiety Test

Defensive burying model. The defensive burying (DB) model has been used to screen-proposed antianxiety compounds (41–43) and for the assessment of changes in animal emotionality associated with various normally occurring conditions (12,13,34,35) and laboratory manipulation (36). As described by Treit (1985), the paradigm was classified into the group of "phylogenetically prepared" animal models. The paradigm is different from the classical conditioning one or operant paradigms in that burying behavior is observed each and every time the animal with no previous behavioral training faces an aversive stimulus (41–43). Rats show defensive behavior when facing aversive stimuli such as electric shock, odors, toxic substances, or light sources (41–43). The behavior consists of a series of stereotyped movements of the forepaw and the entire body aimed to cover the electrode with fine sawdust from the bottom of the cage through which the animal receives, each time it is touched, a low nonpainful electric shock of 0.3 mA (10–13,17,18,34–36,43). During the burying behavior test, the animal is placed for 10 min in an acrylic cage (27 × 16 × 23). The electric shock was delivered from a stimulator (Grass Medical Instruments, Model 54JR, Quincy, MA) through bipolar electrode, 7 cm in length, attached to the wall of the cage (43). Once the electrode was identified as the source of the aversive stimulus, the animals displayed the DB behavior. The recorded parameters were: 1) latency of burying, i.e., time elapsed between the shocks and defensive burying; 2) the cumulative time the rats showed defensive burying; and 3) the number of received shocks (43). The expression of defensive burying behavior has been considered to reflect the rat's emotional level in a direct way i.e., low defensive burying equals diminished experimental anxiety. On the contrary, high burying behavior indicates increased experimental anxiety (41–43). The latency of burying has been related to the ability of animals to identify the electrode as the source of the aversive stimulus (41). The amount of shocks received may indicate putative nocieceptive changes induced by pharmacological treatment (42,43).

Social interaction test. The social interaction model has also been related to the group of "phylogenetically prepared" animal paradigms, and has been successfully used both for screening drugs with anxiolytic potential in clinics (14–16,19) and for the study of the neurobiology of anxiety in rats (34,36). The model includes three different aversive elements: photophobia, a clean, smooth area, and the encountering with an unacquainted conspecific partner. Rats in the wild condition are nocturnal animals. This provides them with adaptive advantages such as being aware of diurnal predators. Thus, increased emotional tonus can be observed when, for experimental needs, an animal is placed in an illuminated area, which simulates a vulnerable situation. On the other hand, it has been reported that rats perceive most information of the surroundings through smelling. Thus, the lack of essential information keeps the rat unaware of putative dangerous places. The sudden encounter with an unknown test partner, after being isolated for 72 h (see below), represents an additional aversive element because the animal cannot anticipate the behavior of conspecific. These three aversive elements lose their stressful nature as the test develops becoming almost neutral at the end of a 15-min social interaction test (14–16,19,34,36). In this paradigm, two unacquainted males with a weight difference of no more than 10 g, are placed to interact with each other in a highly illuminated, smooth, clean arena with no odorous background. The arena is surrounded by an acrylic cylinder of 68 × 48 cm. During the social interaction, spontaneous behaviors such as sniffing, nipping, grooming, kicking, boxing, wrestling, jumping on, and crawling on or over the partner are recorded. Once the animals finish the social in-

teraction test, they are transported to a contiguous experimental room and defensive burying measures are carried out.

Drugs

Hoffmann–LaRoche, Basel, Switzerland, and Silanes Labs., México, generously provided flumazenil (FL) and diazepam (DZ), respectively. Flumazenil was dissolved in distilled water and Tween 80 (1 drop per 1 ml). Diazepam was suspended in a 0.2% methylcellulose solution. The drugs were administered intraperitoneally (IP) at a volume of 2 ml/kg, 30 min prior to the DB test.

Procedure

Animals were kept individually in acrylic cage ($27 \times 16 \times 23$) during 72 h previous to experimental sessions. The behavioral tests were performed 2 h after dark period started, and took place in a room illuminated with red light, contiguous to the housing chamber. The subjects were gently removed from the cage and placed into the social interaction arena with an unknown partner, and remained there for as long as described for each experimental design. The burying behavior assay was carried out after social interaction, except for those groups used as universal or particular controls (see below). Subjects were randomly selected for each experimental design. Each group, in each experiment, was conformed by an independent number of animals submitted to analogous conditions. The results obtained were analyzed by means of the Kruskal–Wallis ANOVA followed by the Mann–Whitney *U*-test (39).

Experiment 1. Putative Effect of Flumazenil on Defensive Burying Facilitation Induced by a Short, 1.5-Min Social Interaction Experience

To explore the role of the BZ receptor on a 1-1/2-min social interaction experience-induced defensive burying facilitation, the antagonist to this receptor, flumazenil was injected IP at 2.5, 5, and 10 mg/kg. Briefly, 28.5 min after flumazenil injection, pairs of unacquainted male rats were placed in a smooth, clean, and illuminated area surrounded by an acrylic cylinder, and the social interaction behavioral repertoire was recorded. Immediately after the 1.5-min social interaction experience, defensive burying was measured in flumazenil-treated animals (2.5, 5, and 10 mg/kg). The rational of the described design was to begin defensive burying study at the maximum flumazenil time effect (30 min). To study the possibility of flumazenil (10 mg/kg)-elicited actions on defensive burying per se, a group of animals was treated and tested for burying behavior without social interaction experience. Two additional groups were treated with saline solution or vehicle 28.5 min before the social interaction test (1.5 min) and defensive burying were carried out. To determine if the IP saline injection had an effect per se, a group of animals was injected, and defensive burying test was performed 30 min after the manipulation. Finally, a group of animals was tested for defensive burying without manipulation.

Experiment 2. Putative Effect of Flumazenil on Defensive Burying Inhibition Induced by a Long, 15-min Social Interaction Experience

To explore the role of the BZ receptor on 15 min of social interaction experience-induced defensive burying inhibition antagonist of this receptor, flumazenil was injected IP at 2.5, 5, and 10 mg/kg. Briefly, 15 min after flumazenil injection pairs of unacquainted male rats were placed in a smooth, clean, and illuminated area surrounded by an acrylic cylinder, and the social interaction behavioral repertoire was recorded. Immediately after the 15 min of social interaction experience, defensive burying was measured in flumazenil-treated animals (flumazenil groups; 2.5, 5, and 10 mg/kg). The rational of the described design was to begin defensive burying study at the maximum flumazenil time effect (30 min). To explore the possibility that flumazenil (10 mg/kg) exerts an effect on defensive burying per se, a group of animals was treated and tested for burying without social interaction experience. Two additional groups were treated with saline solution or vehicle 15 min before social interaction test, and studied for defensive burying after long social experience (15 min). To determine if the IP saline injection had effect per se, a group of animals was injected, and defensive burying test was carried out 30 min later. Finally, a group of animals was tested for burying behavior without manipulation.

Experiment 3. Putative Effect of a Suboptimal Dose of Diazepam on Defensive Burying Facilitation Induced by a Short, 1.5-min Social Interaction Experience

To explore the role of the BZ receptor on a 1-1/2 min social interaction experience-induced defensive burying facilitation, the agonist to its receptor, diazepam, at a suboptimal dose, was IP injected at 0.25 mg/kg. To determine this suboptimal dose, the effect of three diazepam doses (0.25, 0.5, and 1.0 mg/kg) on burying behavior were tested. Briefly, pairs of unacquainted male rats were placed in a smooth, clean, illuminated area surrounded by an acrylic cylinder, and the social interaction behavior repertoire was recorded. Diazepam was administered IP 28.5 min before a short social interaction experience and immediately after defensive burying assay was performed. The rational of the described design was to begin the burying behavior study at the maximum diazepam time effect (30 min). Two additional groups were treated with saline solution or vehicle 28.5 min before the social interaction test, and studied for defensive burying after a brief social interaction experience. To determine if the IP saline injection had effect per se, a group of animals was injected, and defensive burying test was carried out 30 min after the manipulation. Finally, a group of animals was tested for defensive burying without manipulation.

RESULTS

Experiment 1. Effect of Flumazenil on Defensive Burying Facilitation Induced by a Short, 1.5-Min Social Interaction Experience

Figure 1 shows the effect of three doses of flumazenil (2.5, 5, and 10 mg/kg) on defensive burying levels elicited by a short social interaction experience (Kruskal–Wallis ANOVA, $H(8) = 18.813$, $p \leqslant 0.01$). These animals showed an important increase of cumulative defensive burying of 67% compared to the control–control group (Fig. 1A). This increasing effect was blocked by saline or vehicle injection in 44.7 and 42.1%, respectively (Fig. 1B). In addition, the administration of flumazenil increases, in a dose-dependent manner, the blocking action of saline administration in 73.6%, comparing short exposure to social interaction with 10 mg/kg flumazenil group (Fig. 1B). Interestingly, defensive burying levels of rats treated with 10 mg/kg flumazenil were lower than those ob-

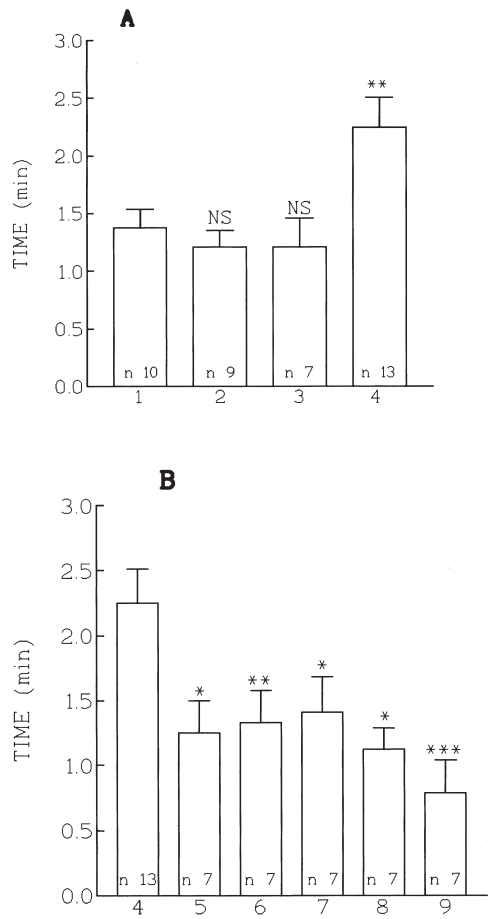

FIG. 1. Effect of flumazenil on facilitated defensive burying elicited by a brief, 1.5 min social interaction experience. The bars represent the mean time ± SE in minutes of the cumulative burying behavior as follows: (A) control groups; 1) control–control; 2) control–saline; 3) flumazenil–control; 4) group submitted to social interaction for 1.5 min. (B) Groups tested under different experimental conditions, all of them experiencing 1.5 min of social interaction; 4) as described above; 5) saline-treated; 6) vehicle-treated; 7) flumazenil 2.5 mg/kg; 8) flumazenil 5 mg/kg, and 9) flumazenil-treated group, 10 mg/kg. Mann–Whitney *U*-test, NS = nonsignificant; $*p \leq 0.05$, $**p \leq 0.01$; $***p < 0.001$.

served for control–control by 57.4% and for saline and vehicle groups, by 28.9 and 31.5%, respectively (Fig. 1A and B). The groups treated with 2.5 and 5 mg/kg of flumazenil showed defensive burying levels similar to control animals (Fig. 1B). Flumazenil per se and saline IP-treated groups showed no change in burying behavior values (Fig. 1A). No changes were observed in latency of defensive burying (Kruskal–Wallis ANOVA, H (8) = 7.185, NS), or in the number of electric shocks received (Kruskal–Wallis ANOVA, H (8) = 8.656, NS; Table 1). None of the experimental procedures altered the amount of social interaction behaviors (Kruskal–Wallis ANOVA, H (5) = 8.411, NS; Table 1).

Experiment 2. Effect of Flumazenil on Defensive Burying Inhibition Induced by a Long, 15-min Social Interaction Experience

Figure 2 shows the effect of flumazenil at 2.5, 5, and 10 mg/kg on defensive burying inhibition elicited by large social interaction experience; Kruskal–Wallis ANOVA, $H(8) = 41.322$, $p \leq 0.001$). The rat group that had experienced 15 min of social interaction showed diminished defensive burying levels by 78.3%; control–control vs. 15 min of social interaction group (Fig. 2A). This effect was reverted by the saline or vehicle, by 59.3 and 67.5%, respectively (Fig. 2B). The animal group treated with 10 mg/kg of flumazenil magnified, in a dose-dependent manner, the partial blocking effect of the injection on defensive burying in rats exposed to 15 min of social interaction by 85.8% (Fig. 2B). Interestingly, flumazenil, at 10 mg/kg, induced defensive burying values above the control–control group, of 35.5% (Fig. 2A and B). It is interesting to note that burying behavior levels in groups treated with flumazenil at 2.5 and 5 mg/kg were similar to the control group (Fig. 2A) compared to the control–control group. In this experiment, the amount of social interaction at the end of the social interaction test reached minimal statistic significance (Table 2; Kruskal–Wallis ANOVA, H (5) = 11.497, $p \leq 0.05$). No changes were observed in the latency of defensive burying (Kruskal–Wallis ANOVA, H (8) = 4.170, NS), or in the number of received electric shocks by long exposure to social interaction animals (Kruskal–Wallis ANOVA; $H(8) = 8.876$, NS; Table 2).

TABLE 1

EFFECT OF FLUMAZENIL IN THE MEAN TIME ± SE IN SECONDS ON SOCIAL INTERACTION (SI), LATENCY OF DEFENSIVE BURYING IN MIN (LDB), AND NUMBER OF ELECTRIC SHOCKS RECEIVED (ES)

	SI	LDB	ES
1. Control–control (n = 10)	—	0.92 ± 0.14	2.60 ± 0.45
2. Control–saline (n = 9)	—	1.00 ± 0.18	3.11 ± 0.65
3. Flumazenil–control (n = 7)	—	0.78 ± 0.07	2.85 ± 0.45
4. 1.5 min of social interaction (n = 13)	25 ± 4	0.84 ± 0.11	4.07 ± 0.54
5. saline-treated (n = 7)	27 ± 2	1.11 ± 0.42	4.14 ± 0.79
6. vehicle-treated (n = 7)	34 ± 2	0.63 ± 0.10	3.00 ± 0.30
7. flumazenil 2.5 (n = 7)	20 ± 3	1.46 ± 0.40	4.14 ± 0.59
8. flumazenil 5.0 (n = 7)	25 ± 1	0.77 ± 0.16	3.85 ± 0.34
9. flumazenil 10.0 (n = 7)	27 ± 5	1.15 ± 0.20	3.28 ± 0.56

In groups 4–9 the social interaction test lasted 1.5 min. The groups are presented as follows: 1) Control–control; 2) Control–saline; 3) flumazenil–control; 4) group submitted to social interaction experience for 1.5 min; 5) saline-treated; 6) vehicle-treated; 7) flumazenil-treated 2.5 mg/kg; 8) flumazenil-treated 5 mg/kg, and 9) flumazenil-treated group 10 mg/kg.

Experiment 3. Effect of a Suboptimal Dose of Diazepam on Defensive Burying Facilitation Induced by a Short, 1.5 min Social Interaction Experience

Figure 3 shows the effect of the suboptimal dose for 0.25 mg/kg diazepam on defensive burying levels in animals experiencing short social interaction experience (Kruskal–Wallis ANOVA, H (8) = 32.026, $p \leq 0.001$). Animals treated with a suboptimal dose of diazepam 0.25 mg/kg, showed reduced defensive burying levels, amplifying the saline-induced decrease in burying behavior by 78.9% (short social interaction submitted vs. diazepam 0.25 group; Fig. 3B). Defensive burying was considerably below the levels exhibited by the control animal group by 73.3% (Fig. 3A). The levels of the saline and vehicle groups were different to the values exhibited by the 0.25 mg/

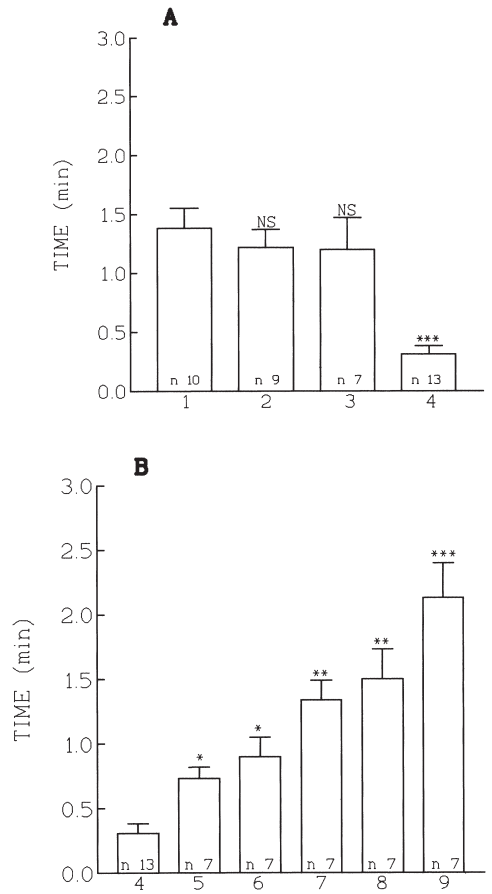

FIG. 2. Effect of flumazenil on the inhibition in defensive burying elicited by a long, 15 min social interaction experience. The bars represent the mean time ± SE in minutes of the cumulative burying behavior as follows: (A) control groups, 1)control–control; 2) control–saline; 3) flumazenil–control; 4) group submitted to social interaction for 15-min. (B) Groups tested under different experimental conditions, all of them experiencing 15 min of social interaction, 4) as described above; 5) saline-treated; 6) vehicle-treated; 7) flumazenil 2.5 mg/kg; 8) flumazenil 5.0 mg/kg, and 9) flumazenil-treated group 10 mg/kg. Mann–Whitney *U*-test, NS = nonsignificant; $*p \leq 0.05$, $**p \leq 0.01$; $***p < 0.001$.

kg of diazepam-treated group, i.e., 62.7 and 56.7% higher, respectively (Fig. 3B). Diazepam, at 1 mg/kg, effectively reduced defensive burying (Fig. 3A), while 0.5 and 0.25 mg/kg did not reduce burying behavior levels (Fig. 3A). No changes were observed in the latency of defensive burying or in the number of electric shocks received and the amount of social interaction behavior (Kruskal–Wallis ANOVA, H (8) = 11.807, NS; H (8) = 9.498, NS, and H (3) = 2.891, NS, respectively).

TABLE 2

EFFECT OF FLUMAZENIL IN THE MEAN TIME ± SE IN SECONDS OF SOCIAL INTERACTION (SI), THE LATENCY OF DEFENSIVE BURYING IN MIN (LDB), AND THE NUMBER OF ELECTRIC SHOCKS RECEIVED (ES)

	SI	LDB	ES
1. Control–control (n = 10)	—	0.92 ± 0.18	2.6 ± 0.45
2. Control–saline (n = 9)	—	1.00 ± 0.18	3.11 ± 0.65
3. Flumazenil–control (n = 7)	—	0.78 ± 0.07	2.85 ± 0.45
4. 15 min of social interaction (n = 13)	121 ± 14	1.95 ± 0.66	3.30 ± 0.52
5. saline-treated (n = 7)	178 ± 8*	0.71 ± 0.15	2.14 ± 0.34
6. vehicle-treated SI (n = 7)	144 ± 28	1.31 ± 0.38	2.42 ± 0.20
7. flumazenil 2.5 (n = 7)	157 ± 11	1.13 ± 0.21	3.14 ± 0.45
8. flumazenil 5.0 (n = 7)	129 ± 16	0.85 ± 0.11	3.28 ± 0.35
9. flumazenil 10.0 (n = 7)	126 ± 26	0.98 ± 0.13	4.00 ± 0.80

In groups 4–9 the social interaction experience lasted 15 min. The groups are presented as follows: 1) Control–control; 2) Control–saline; 3) flumazenil–control; 4) group submitted to social interaction experience for 15 min; 5) saline-treated; 6) vehicle-treated; 7) flumazenil-treated 2.5 mg/kg; 8) flumazenil-treated group 5 mg/kg, and 9) flumazenil-treated 10 mg/kg. Mann–Whitney *U*-test, $*p \leq 0.05$ comparison between four and five groups.

DISCUSSION

The main findings obtained in the present work were: 1) a dose-dependent flumazenil (2.5, 5, and 10 mg/kg) blocking effect on defensive burying in animals with a short exposure to social interaction, an action that amplified injection-induced defensive burying reduction (Fig. 1B). 2) A dose-dependent flumazenil (2.5, 5, and 10 mg/kg)-reverting effect on defensive burying in animals with a longer exposure to social interaction experience, which enhanced the injection-elicited defensive burying increase (Fig. 2B). 3) An increase of injection-elicited defensive burying reduction in experienced rats with short social interaction after the administration of a suboptimal dose of diazepam (0.25 mg/kg; Fig. 3B). The ANOVA test revealed significance for the amount of social interaction in animals with a long exposure (Experiment 2, Results section) but not in the briefly exposed group (Experiment 1 and 3, Results section). This last effect could be related to the minimal time rats spent in the social interaction arena (Tables 1 and 3). The paired comparison of social interaction levels in the long exposed group revealed significance only in saline animals that showed an increased amount of social behaviors, while the vehicle group did not (Table 2). It is interesting to note that flumazenil-treated groups showed social interaction levels similar to those observed for the 15 min of social interaction animals (Table 2). No changes in the latency of burying behavior and in the number of electric shocks received were observed in Experiments 1, 2, and 3 (Tables 1, 2, and 3). This evidence could sustain that social interaction-induced transient effects on burying behavior were related, specifically to changes in rat emotional responses.

The present work replicated data published previously by our group regarding transient defensive burying effects elicited by social interaction experience (36) and saline injection [Figs. 1, 2, and 3; (34,38)]. According to the evidence, facilitated defensive burying was observed in animals with short social interaction experience [Figs. 1 and 3; (36)]. In addition, results showed that a prolonged social interaction period elicited lower defensive burying values in subjects with a long social interaction exposure [Fig. 2; (36)]. To elucidate the temporal course of facilitated defensive burying, a group of animals experiencing a short social interaction experience was removed from the arena remaining undisturbed in their home cages for 15 min, and defensive burying measured (36). We observed that facilitated burying behavior decreases to basal levels 15 min after social interaction ceased (36). We

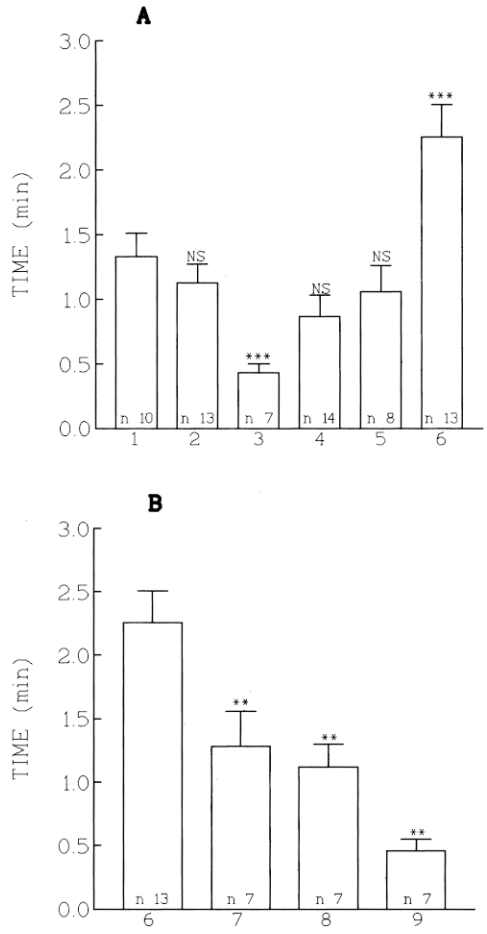

FIG. 3. Effect of diazepam at a suboptimal dose (0.25 mg/kg) on facilitated defensive burying elicited by a brief, 1.5-min social interaction experience. The bars represent the mean time ± SE in minutes of the cumulative defensive behavior as follows: (A) control groups; 1) control–control; 2) control–saline; 3) diazepam 1 mg/kg; 4) diazepam 0.5 mg/kg; 5) diazepam 0.25 mg/kg; 6) group submitted to social interaction for 1.5 min. (B) Groups tested under different experimental conditions all of them experiencing 1.5 min of social interaction; 6) as described above; 7) saline-treated; 8) vehicle-treated, and 9)diazepam 0.25 mg/kg group. Mann–Whitney *U*-test, NS = nonsignificant; $^{**}p \leq 0.01$; $^{***}p < 0.001$.

have studied the temporal course effect of decreased experimental anxiety observed in rats submitted to a long social interaction experience as well. This was achieved by having subjects that had 15 min of social interaction to rest quietly in their own home cages for 15 or 30 min and thereafter tested in the defensive burying paradigm (36). This experiment revealed that burying behavior returns slowly to control levels 30 min after the social interaction finished (36). Thus, evidence showed that the social interaction experience-induced changes in defensive burying are transient, and inversely related to the time the rat spent within the social interaction arena (36). An analogous complex biphasic modulation of emotional responses has been proposed in chronic stress and glucocorticoid levels, inducing enhanced or depressed hippocampal function, depending on, among other factors, the duration of exposure to stressful environmental stimuli (26). Additional evidence regarding the specificity of social interaction-elicited emotional changes is also supported by results presented in the current experiments. Thus, the fact that no differences in burying behavior latency were observed, a parameter that reflects the animal's ability for associative processes (Tables 1, 2, and 3), suggests that animals were able to identify the electrode as the source of aversive stimuli.

TABLE 3

EFFECT OF DIAZEPAM IN THE MEAN TIME ± SE IN SECONDS OF SOCIAL INTERACTION (SI), LATENCY OF DEFENSIVE BURYING IN MIN (LDB), AND THE NUMBER OF ELECTRIC SHOCKS RECEIVED (ES)

	SI	LDB	ES
1. Control–control ($n = 10$)	—	0.92 ± 0.14	0.60 ± 0.45
2. Control–saline ($n = 13$)	—	0.67 ± 0.12	3.00 ± 0.22
3. diazepam–control 1.0 ($n = 7$)	—	1.51 ± 0.36	4.85 ± 0.50
4. Diazepam–control 0.5 ($n = 14$)	—	1.33 ± 0.28	4.50 ± 0.27
5. diazepam–control 0.25 ($n = 8$)	—	1.06 ± 0.12	4.00 ± 0.37
6. 1.5 min of social interaction ($n = 13$)	25 ± 4	0.84 ± 0.61	4.07 ± 0.54
7. saline-treated ($n = 7$)	27 ± 2	1.11 ± 0.42	4.14 ± 0.79
8. vehicle-treated ($n = 7$)	21 ± 2	0.85 ± 0.18	4.14 ± 0.59
9. diazepam 0.25 ($n = 7$)	26 ± 2	1.80 ± 0.61	1.85 ± 0.26

In groups 6–9 the social interaction experience lasted 1.5 min. The groups presented are as follows: 1) control–control; 2) control–saline; 3) diazepam–control 1.0 mg/kg; 4) diazepam–control 0.5 mg/kg; 5) diazepam–control 0.25 mg/kg; 6) group submitted to social interaction experience for 1.5 min; 7) saline-treated; 8) vehicle-treated; 9) diazepam 0.25 mg/kg group submitted for 1.5 to the social interaction arena.

The defensive burying changes observed in saline and vehicle-treated groups (Figs. 1, 2, and 3), also replicates evidence obtained in our laboratory (34,38). These reports showed that injection-induced defensive burying increases when the interval between injection and burying behavior assay was short (1.5 min), and inhibited when the interval was slightly longer (3 min). The temporal course analysis of the injection-induced changes in defensive burying showed that subjects tested for burying behavior 30 min after saline-injection exhibited defensive burying values similar to those of the control animals, an effect repeated in the present work [see control–saline group Figs. 1, 2, 3; (34)]. The iterative saline injection with an interval between manipulations of 13.5 min elicited defensive burying decrease below the control values, while a longer interval, 28.5 min, reduced burying behavior to control levels compared to rats receiving a single saline injection and tested 1.5 min later (34). This evidence supports the notion that rats are protected against anxiety when two aversive stimuli are delivered; completely with an interval of 13.5 min, and partially when the interval was 28.5 min (34). Furthermore, the injection-elicited protection against defensive burying increase in rats with short exposure was reported recently (38). In this work, saline injection elicited decreased defensive burying below control values in animals with short social interaction exposure when the anxiety test was carried out 5 and 15 min after injection (38). Burying behavior similar to control values was observed 30 min after saline injection, an effect analogous to that observed in the present work [Figs. 1 and 3; (38)]. Animals tested 45 min after injection showed a clear tendency towards burying facilitation, as high as in the group with brief social interaction exposure, but no statistical difference vs. control animals was observed when paired comparisons were performed (38). Jointly, this evi-

dence might explain why, in the present experiments, saline or vehicle injection in animals with a brief social interaction experience blocked the anxiogenic-like effect (Figs. 1 and 3). In addition, a partial blocking effect was also seen in animals with a long social interaction exposure. This action could be explained with the evidence that at 15 min after injection, defensive burying exhibited values similar to those observed for control groups (34). This evidence suggests that in both cases, brief and long social interaction experiences failed to change defensive burying when injection was previously delivered, impeding social interaction experience from modifying defensive burying [Figs. 1, 2, and 3; (34,38)]. Therefore, flumazenil and a putative endogenous ligand (one or more), activated by injection, eventually modulating the BZ receptor function, can be assumed. This additive action finally amplified the effect of flumazenil, protecting the animal against experimental anxiety (Figs. 1 and 3) or reducing the anxiolytic-like action (Fig. 2). It is important to note that flumazenil itself was unable to promote defensive burying changes in naive animals, suggesting that the compound was only able to interact on the activated BZ receptor. These actions could be explained on the basis of one of the classical dogmas of pharmacology "...the action of an antagonist can be observed only in the presence of its agonist and lacking actions per se" (22).

It is interesting to note the increased social interaction behaviors in saline-injected animals (Table 2). In these experiments social interaction levels increase by 47% compared to the 15-min social interaction-experienced noninjected group (Table 2). This evidence provides an additional support to the hypothesis that previous aversive stimulus protect against anxiety if an adequate interval is observed between delivering the stressor and the anxiety test (34,36,38). The idea that increased social interaction reflects the anxiolytic state has been previously proposed (15,16,19). The participation of the BZ receptor mediating increased social interaction elicited by saline injection can be suggested by the fact that flumazenil blocked this effect in the 5.0 and 10 mg/kg groups (Table 2). However, we have to mention that we did not observe facilitated social behaviors in the vehicle-treated group (Table 2). This failure could be explained on the basis that these findings should be considered as marginal, because our original purpose was to study changes in defensive burying elicited by social interaction (see Introduction). Up to now, we have not characterized the temporal course effect of saline injection-elicited changes in social interaction. Thus, we ignore, if in this particular case emotional response behaves in a biphasic mode, as defensive burying behaved. Further experiments should elucidate these points.

Results obtained in various laboratories reporting that preceding stressful experiences induce increased emotional rat responses agree with findings obtained in the present work showing increased defensive burying in the short social interaction-experienced group [Figs. 1 and 3; (7,8,19,34,36,46)]. By contrast, anxiety-protecting actions elicited by several experimental and physiological conditions in female and male rats have been reported (11,13,34–36,38). These facts agree with findings of the present work that show decreased burying behavior levels in long social interaction experienced rats (Fig. 2). The apparent controversy among the above-presented data might be related to the fact that the rat emotional response presents a biphasic pattern of expression, observed when the temporal course of the aversive behavior is studied.

In addition, changes in BZ receptor affinity elicited by a stressful experience have also been reported (19,27,32). Thus, bimodal changes (down-and up-regulation) in frontal cortex and hippocampal rat brain areas after cold swimming was observed (27). Moreover, data showing increased $GABA_A$ receptor function i.e., chloride-augmented currents, enhanced chloride synaptosomal uptake, and BZ increased affinity in social interaction-naive rats exposed for 7.5 min compared to experienced animals, have been reported (32). The evidence supports the idea that there is a turning point between the decreased–increased function of the $GABA_A$ receptor complex (32). Furthermore, recently, BZ desensitization elicited by previous experience to the elevated plus-maze for 5 min has also been presented (18). In these experiments, authors report the lack of anxiolytic action of midazolam in experienced animals and a flumazenil reinstalling effect when coadministered with midazolam. In addition, in this report, an anxiolytic effect of flumazenil per se in a second trial was also observed, suggesting that the role of a putative negative benzodiazepine modulator is responsible for decreased BZ receptor function. These actions recall the effect of flumazenil impeding social interaction-induced defensive burying levels, i.e., decreasing the emotional response in animals with short social interaction exposure (Fig. 1B). Previously, flumazenil has been used for its ability to block benzodiazepine behavioral actions in animals (28,40), to prevent stress-induced release of biogenic amines (31) and to revert the effects of benzodiazepine overdoses under clinical conditions (4,5,21). The present evidence (Figs. 1, 2, and 3) provides support to the hypothesis of the benzodiazepine receptor mediation in facilitated defensive burying in animals with a short social interaction exposure (Figs. 1 and 3) and in the burying behavior inhibition in animals with a long social interaction experience (Fig. 2).

Several compounds could be proposed to act at the benzodiazepine receptor level, mediating the transient responses. Among them, the benzodiazepine inhibitory peptide (DBI) able to reduce receptor activity and diazepam binding (2), could be involved in facilitated defensive burying (Figs. 1 and 3). In addition, anxiogenic actions of sulfated progesterone derivative have been recently reported (25,30,33). On the other hand, anxiolytic effects elicited by 5-α reduced metabolites of progesterone, progesterone itself (17,25), and corticosterone (30,33) have been reported. This evidence supports the possibility of negative–positive benzodiazepine modulation acting in a continuum, where decreased functional state increases, depending on the time elapsed after delivering the aversive stimuli. This type of modulation increases animal behavioral adequacy to environmental requirement (6).

In summary, flumazenil enhanced injection-elicited action impeding defensive burying expression in animals with short social interaction exposure (Fig. 1). By contrast, flumazenil amplified the effect of IP saline facilitating defensive burying in animals with long social interaction exposure (Fig. 2). In addition, a suboptimal dose of diazepam enhanced saline-elicited effect on defensive burying reduction in animals with short social interaction exposure (Fig. 3). Jointly, this evidence supports the notion that flumazenil and diazepam actions on defensive burying depend on the BZ receptor functional state. However, further experiments should be undertaken to elucidate the putative participation of other neurotransmitter systems on social interaction-induced transient defensive burying levels.

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