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# Transient Emotional Changes Elicited by Intraperitoneal Saline Injection: Effect of Naloxone and Flumazenil

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SALDÍVAR–GONZÁLEZ, A., ARIAS, C. AND MONDRAGÓN–CEBALLOS, R. *Transient emotional changes elicited by intraperitoneal saline injection: Effect of naloxone and flumazenil.* PHARMACOL BIOCHEM BEHAV **56**(2) 211–220, 1997.—The effect of the intraperitoneal (IP) saline injection was assessed by using the defensive burying (DB) and the elevated plus-maze (EPM) anxiety paradigms in rats. Animals were handled gently by the body, injected IP with saline solution, 2 ml/kg, and tested independently in the defensive burying as well as in the elevated plus-maze test at different times after the IP injection: 1.5, 3, 5, 10, 15, and 30 min. A transient effect of IP saline injection was observed (i.e., increased DB in animals tested 1.5 min after injection) and a decrease in this parameter when studied 3 min after the injection. No changes at 5, 10, 15, and 30 min after the injection were found. To discriminate the putative participation of the opiate peptide and benzodiazepine receptors in the actions of the IP injection, flumazenil (5 mg/kg) and naloxone (1 mg/kg) were administered. The increase in DB at 1.5 min was masked by double injection, an effect blocked by naloxone, but not by flumazenil, while both of them reverted the decrease in DB response in animals tested 3 min after injection. A partial action of the IP in the animals tested in the elevated plus-maze test was found. Present results are discussed on the basis of behavioral and pharmacological evidence. 

Anxiety Defensive burying Elevated plus-maze Flumazenil Intraperitoneal saline injection Naloxone

A WIDE variety of manipulations inducing psychoemotional stress in rats have been published in the past. Among others, the IP injection in rats, represents a broadly used technique in pharmacological designs. However, despite the wide use of this procedure in experimental anxiety designs, for preclinical drug screening, the intrinsic action of IP injection on the animal's emotional tonus, as well as the temporal course of this action have not been adequately assessed. Some difficult points in such an approach should be emphasized. The injection by itself as a laboratory technique might be divided into a two steps procedure. The first is the necessary handling of the animal to inject it, followed by the needle puncturing event that stimulates the rat ventral skin, muscles and peritoneum pain receptors. As an evidence of IP induced stress, we can mention the recent finding that the injection of saline induces changes in cAMP levels in the brain cortex of rats (52).

Two neurotransmitter systems have been consistently involved in handling and other nociceptive procedures in rats: the GABA-benzodiazepine, GABA-Bz (2–4,10,25), and the opiate peptide systems (5,12,26,28,44,51,58). Consequently, several lines of evidence have shown a role of the GABA-Bz receptor in mediating the response to acute or chronic handling (2,3,10), for example, a rapid reduction of GABA-Bz binding affinity in the rat's frontal cortex as a response of acute handling and, consequently, a protective action against reduction in GABA-Bz binding values in the frontal cortex produced by chronic handling, compared with acute handled animals (4). Furthermore, chloride flux increases in synaptosomes in animals habituated to handling when compared with naive rats (10). Moreover, the actions of several manipulations inducing reduction in the Bz receptors numbers, such as foot electric shock or post-natal isolation of rat pups (7), support the notion of the involvement of Bz receptors in stress-induced procedures. However, some controversy on the relationship between Bz

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binding with stress-induced responses has arisen. Increased Bz binding has been observed in brain tissue of rats submitted to swimming at different water temperatures (37,49) as well as when submitted to immobilization stress (7). On the other hand, decreased levels in the number of Bz receptors in frontal cortex and hippocampus have been observed in rats prior to ejaculation, followed by a rapid increase immediately after ejaculation (45). From the above referred findings, the bidirectional modality of stress-inducing changes and the physiological states of the GABA-Bz level depends on the nature of the stressor (2,7) and of regulatory actions (45), suggesting that two opposite effects are mediated through the GABA-Bz system, i.e., positive or negative modulation (2,7,45).

Participation of opiate peptides mediating stress-induced responses has also been reported (51). Thus food and water deprivation induced alterations in opiate binding have been reported (5,28,51,58). Restraint-induced reduction in [$^3$H]etorphine binding in rat brain homogenates (26), reduction in leu/enkephalin binding elicited by swimming (12) and acute intermittent foot shock, conditioned fear (11) have also been described. Besides, stress-induced actions on the number of opiate receptor in brain tissue, and the effect of pain and stress induced antinociceptive responses have been pointed out (24,33), as well as the action of foot shock on the nociception measured by tail-flick latencies (51). All of this evidence supports the notion that the opiate system participates in pain-induced experimental anxiety responses.

In addition, other neurotransmitter systems have been associated to mediation of anxiety response in rats. Thus, evidence on the participation of dopamine (18,31,32), norepinephrine (1,34,41), and serotonergic neurotransmitter systems (8,29,30, 36) have been reported.

The actions of endogenous ligands (15,25) participating in the mediation of aversive responses in animals exposed to stressful stimuli have been reported. Thus, the induction of aversive behaviors by a foregoing situation such as the exposure to the elevated plus-maze (14), presentation of a predator odor to rats (63), facing of an unknown partner in an open, highly illuminated, arena (40) or an unfamiliar environment (40) have been described. Recently, data obtained in our laboratory revealed a bimodal effect on defensive burying, observed in rats after exposure to an unknown partner in an open, highly illuminated, arena (47), or in thirsty rats and water satiated animals in a water enforced design (46). These results support the idea that the bidirectional modulation in the GABA-Bz receptor system might mediate the bimodal nature of behavioral responses.

The present work assesses the action of the IP saline injection on defensive burying and elevated plus-maze test at different times following injection. Additionally, the participation of opiate and benzodiazepine receptor systems on the putative effect of the IP injection on defensive burying was also studied.

## GENERAL METHOD

### *Animals*

Male Wistar rats weighing 250–300 g were used in the experiments. The animals were maintained in an inverted light-dark cycle (light off 1000–2200 h), with free access to food and water. Animals were housed in groups of six in jumbo size (55 × 35 × 20 cm) acrylic cages. Seventy-two hours prior to the experiments the rats were moved to individual home cages (27 × 16 × 23 cm), where they remained until the experiments were performed. The experiments started 2 h after onset of the dark phase.

### *Anxiety Tests*

*The defensive burying test.* The defensive burying paradigm is a model well known for its ability to reveal both anxiogenic and antianxiety actions elicited by drugs in rats (55–57) or as a response to a certain physiological state (20–22,46,47) which makes this model suitable to study the neurobiology of stress in rats. The paradigm is based on the natural aversion, expressed as DB, that wild and laboratory rats exhibit when confronted to different aversive stimulus. The paradigm consists physically in an acrylic individual home cage (27 × 16 × 23 cm), with an electrode attached to a prod emerging from a wall of the cage. Through the electrode the rat receives a low electric shock (0.3 mA) each time the prod is touched. The floor cage is covered with fine sawdust, that the animal uses to bury the electrode after the shock. The defensive burying behavior is clearly identified by the stereotyped movements performed by the animal with the paws to cover the prod. The variables recorded are: the latency to show the burying behavior, the burying behavior observed in a 10-min period, and the height of the sawdust pile at the end of the test (55).

*The elevated plus-maze test.* The elevated plus-maze test belongs to the family of phylogenetically determined anxiety models used for the development of putative anxiolytic compounds. Physically the paradigm consists of an elevated, 50 cm, plus-shaped maze, with two 50 × 10 × 50 cm enclosed arms, and the other two 50 × 10 cm arms are open each with an open roof. The paradigm is based on the natural rat aversion to open high places. In this test the time the animal spends in the open arm section is recorded as well as the number of transitions the subject performs from one closed arm section to another during a five min duration test (35,36).

### *Drugs*

The following drugs were used in the present work: naloxone (Sigma Chemicals, St. Louis, MO) and flumazenil (Hoffmann-La Roche, Mexico City). Naloxone (1 mg/kg) was dissolved in saline solution and injected intraperitonealy (2 ml/kg). Flumzenil (5 mg/kg) was dissolved in a distilled water and Tween 80 (1 drop per 1 ml) solution, and administered IP.

### *Statistics*

The burying behavior data were analyzed by means of the Kruskal Wallis ANOVA test. Paired comparisons among means were done using the Mann Whitney U test (48,50).

### *Experiment 1: The Putative Temporal Course Effect of IP Saline Injection on Defensive Burying*

The animals were transported to the experimental room, kindly handled by the body and injected 2 ml/kg of saline solution, after which they were returned to their home cages and left to rest until the anxiety test was carried out. Intervals between the injection and the anxiety test were 1.5, 3, 5, 10, 15, and 30 min. Each of these manipulations were performed on independent groups. Two control animal groups were studied; one of them tested for defensive burying without any prior manipulation; and animals submitted to gently handling before the DB test. The handled group was transported to the room, gently grasped by the body for 32 s (which represents

the mean time the injection lasts) placed in the DB cage and observed either at 1.5 or 3 min after the handling.

*Experiment 2: Effect of Naloxone on the Putative Action of IP Saline Injection on Defensive Burying 1.5 min Afterwards*

In order to elucidate the putative participation of the opiate receptor in defensive burying 1.5 min after the IP injection, the following experiment was carried out. Control animals were handled by the body for 32 s, returned to the home cage and tested for DB 1.5 min after handling was finished. Another group of rats was injected IP with saline solution (2 ml/kg) returned to the home cage and tested for burying behavior 1.5 min after the injection. A third group was IP injected with saline solution twice; the first at time 0, and the second at 13.5 min and tested 1.5 afterwards for defensive burying. The rationale of such a design was to assess if the putative action of the IP injection at 1.5 min, persisted after two injections, since it mimics the way to administer the drug and, on other hand, the behavioral eliciting manipulation. Another group was injected with anloxone (1 mg/kg) and tested 15 min after, in the DB paradigm with the aim to elucidate the effect of naloxone per se on DB. Finally, a group was injected first with naloxone (1 mg/kg, IP) and 13.5 min afterwards with saline solution, and tested for DB 1.5 after saline (and so after 25 min of the naloxone administration) injection.

*Experiment 3: Effect of Flumazenil on the Putative Action of IP Saline Injection on Defensive Burying 1.5 min After*

With the aim of studying the putative mediation of the benzodiazepine receptor on the effect on DB after the 1.5 min injection, the benzodiazepine antagonist, flumazenil, was used in the following experimental design. The control group was kindly held by the body for 32 s, returned to the home cage and tested for burying behavior 1.5 after handling was finished. Another group was injected IP with saline solution (2 ml/kg), returned to the home cage and tested for DB 1.5 after the injection. A double IP saline injection was performed, the first at time 0 and the second 28.5 min afterwards. In order to analyze if flumazenil per se elicits or not changes in DB, one group of animals was injected with flumazenil and tested 30 min later. In another group, flumazenil (5 mg/kg) was firstly injected and 28.5 they were given an injection of saline solution. The aversive response was tested 1.5 min afterwards.

*Experiment 4: Effect of Naloxone on the Putative Action of IP Saline Injection on Defensive Burying 3 min After*

To analyze the putative mediation of opiate receptors 3 min after, the injection procedure, the opiate antagonist, naloxone (1 mg/kg) was administered. Control animals were handled gently for 32 sec and returned to the home cage where they remained during 3 min, after which the DB test was carried out. Another group was injected with saline solution (2 ml/kg), returned to the home cage and tested for burying behavior 3 min after IP injection. The double saline injection control was given: the first injection at time 0, and the second, 12 min later. The animals were tested for DB, 3 min after the second injection (i.e., 15 min after the first one). Another group of animals received naloxone (1 mg/kg, IP) and was tested 15 min later. The experimental group was also injected twice, at time 0 with naloxone (1 mg/kg), and 12 min later, saline solution was injected. These animals were tested 3 min after the saline injection (i.e., 15 min after the naloxone injection).

*Experiment 5: Effect of Flumazenil on the Putative Action of the IP Saline Injection on Defensive Burying 3 min Afterwards*

To assess the putative participation of the benzodiazepine receptor on the effect elicited 3 min after the IP injection of saline solution, the action of flumazenil, the following experiment was performed. The control animal group was gently grasped by the body for 32 s, and returned to the home cage where animals remained undisturbed for 3 min at the end of which the DB was assessed. Another group was injected with saline solution (2 ml/kg), left for a 3 min period in the home cage, and tested for DB once this period elapsed. A third group was injected with saline solution at time 0, and a second saline injection was administered 27 min afterwards. This group was tested for anxiety 3 min after the second injection, (i.e., 30 min after the first injection). To test if flumazenil (5 mg/kg) induced changes in DB, a second group of animals was injected IP and tested after 30 min. The experimental group of animals received flumazenil (5 mg/kg) at time 0, and saline solution 27 min later. The anxiety test was performed 3 min after the second injection had been administered.

*Experiment 6: The Putative Temporal Course Effect of the IP Saline Injection on the Elevated Plus-Maze Test*

The putative action of IP saline injection was studied in the elevated plus-maze test. The animals were grasped gently by the body, injected IP with saline solution (2 ml/kg) and afterwards tested for five min in the elevated plus maze for the following periods: 1.5, 3, 5, 10, 15 and 30 min after the injection. Each of these manipulations were performed on independent groups. Control animals were studied with no manipulation. The measured parameters in the present experiment were: (a) time in open arms; and (b) transitions from one closed arm to the other.

## RESULTS

*Experiment 1: The Temporal Course Effect on Defensive Burying of IP Saline Injection*

Figure 1 shows the effect of the saline solution IP injection on DB. An increase in the mean time of burying behavior in animals tested 1.5 min after injection is observed (control vs handled group: $1.31 \pm 0.16$ vs $1.16 \pm 0.33$, and 1.5 min group $2.12 \pm 0.24$). A decrease in burying behavior in the group tested 3 min after injection is also observed (control vs handled, $1.31 \pm 0.16$ vs $1.32 \pm 0.24$ and 3 min group $0.63 \pm 0.15$). The Kruskal Wallis ANOVA test ($H = 18.620$, $df = 8$, $p \leqslant 0.01$) revealed significant differences between groups. No changes in defensive burying occurred in groups studied at 5, 10, 15, and 30 min after the injection, when compared to control groups. No significant changes in latency to show defensive burying after electric shock were observed ($H = 10.874$, $df = 8$, $p \leqslant 0.20$, NS). Also, no differences were found in the number of electric shocks the animals received in the present experiment ($H = 8.202$, $df = 8$, $p \leqslant 0.41$, NS). The results of both, latency of burying and electric shock, are presented in Table 1. No changes in the height of the bedding material were found (data not shown).

*Experiment 2: Effect of Naloxone on Defensive Burying, 1.5 min After the IP Saline Injection*

Figure 2 shows the effect of naloxone given prior to the IP saline injection on defensive burying. The saline group injected IP twice shows a decrease in DB levels when com-

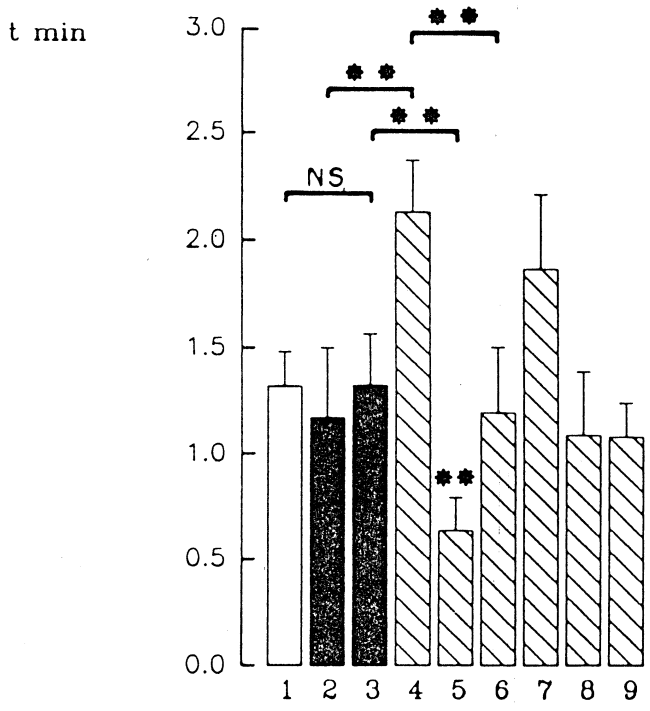


FIG. 1. The temporal course analysis of the effect of the IP injection on defensive burying. Bars represent the mean time ± SE of burying behavior for the following groups: clear bars represent the control not injected group, 1; dark bars represent the control handled not injected groups, 2, 1.5 min; 3, 3 min; the slanted-line bars represent the injected groups at the following times, 4, 1.5 min; 5, 3 min; 6, 5 min; 7, 10 min; 8, 15 min; 9, 30 min. Mann Whitney U test, NS: non significant; $^{**}p \leqslant 0.01$.

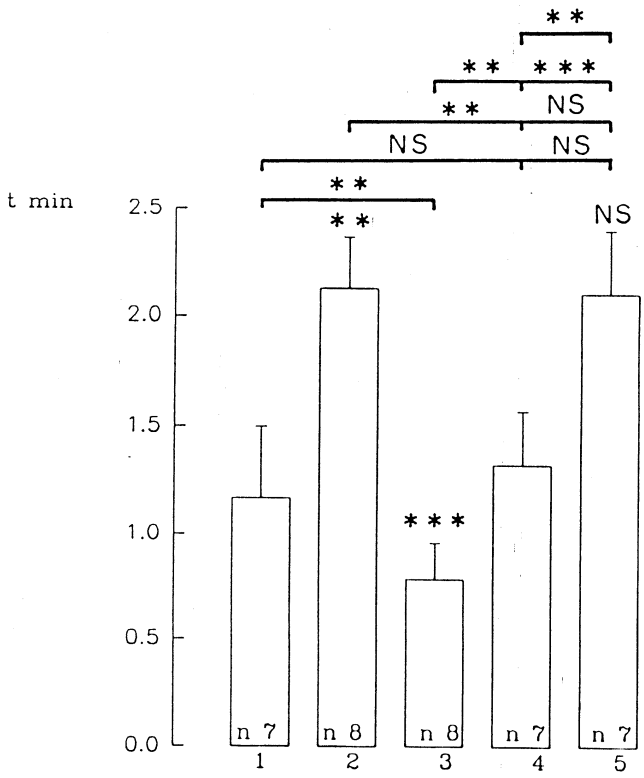


FIG. 2. Effect of naloxone on changes in burying behavior induced by double IP saline injection in animals tested 1.5 after injection. Bars represent the mean time ± SE of DB in the following groups: (1) control group, (2) group tested for anxiety 1.5 min after injection; (3) double saline injected group; (4) naloxone control group; and (5) naloxone experimental group. Mann Whitney U test, NS: non significant; $^{***}p \leqslant 0.0001$; $^{**}p \leqslant 0.02$.

pared to the group injected once (2.12 ± 0.24 vs 0.78 ± 0.17). Naloxone per se lacks actions on defensive burying (1.16 vs 0.33 vs 1.32 ± 0.24), while the experimental group, injected first with naloxone, 13.5 and afterwards with saline solution shows defensive burying levels similar to those observed in the single injection group (2.12 ± 0.24 vs 2.10 ± 0.29). The Kruskal Wallis ANOVA test revealed significant differences (H = 17.475, df = 4, $p \leqslant 0.001$). Table 2 shows the mean latency of burying and the number of shocks received by animal groups in this experiment (H = 3.404, df = 4, $p \leqslant 0.40$, NS and H = 6.243, df = 4, $p \leqslant 0.18$, NS, respectively). No changes in the height of the bedding material were found (data not shown).

TABLE 1

THE TEMPORAL COURSE ANALYSIS OF THE EFFECT OF IP INJECTION ON THE MEAN TIME OF LATENCY OF BURYING BEHAVIOR, AS WELL AS THE MEAN OF SHOCKS RECEIVED AFTER THE IP INJECTION AS DESCRIBED IN EXPERIMENT 1

| Groups | *n* | Latency of DB (min) | No. of Shocks |
|---|---|---|---|
| Control | 12 | 0.66 ± 0.14 | 2.08 ± 0.31 |
| Control handled 1.5 min | 7 | 0.90 ± 0.42 | 2.42 ± 0.36 |
| Control handled 3.0 min | 7 | 1.09 ± 0.21 | 2.42 ± 0.57 |
| 1.5 min after IP | 8 | 1.13 ± 0.38 | 2.00 ± 0.46 |
| 3 min after IP | 7 | 0.87 ± 0.11 | 3.28 ± 0.68 |
| 5 min after IP | 9 | 0.83 ± 0.15 | 2.22 ± 0.32 |
| 10 min after IP | 10 | 1.31 ± 0.19 | 1.70 ± 0.15 |
| 15 min after IP | 7 | 0.78 ± 0.14 | 3.00 ± 0.61 |
| 30 min after IP | 7 | 1.05 ± 0.27 | 2.28 ± 0.42 |

*Experiment 3: Effect of Flumazenil on Defensive Burying, 1.5 min After IP Saline Injection*

Figure 3 shows the effect of flumazenil on the increase in burying behavior observed 1.5 min after IP injection of saline solution. The injected IP saline group twice at 0 and 27.5 min, showed a decrease in defensive burying when compared with the single injected animals (2.12 ± 0.24 ± vs 1.25 ± 0.21). A group injected with flumazenil and tested for DB 30 min afterwards, failed to show changes in DB levels (1.07 ± 0.15). The group injected at time 0 with flumazenil and 27.5 min later with saline failed to revert the decrease in burying behavior (1.59 ± 0.17). The Kruskal Wallis ANOVA revealed significant differences (H = 11.109, df = 4, $p < 0.02$). A control group injected at time 0 with the flumazenil vehiculum (Tween 80, 1 drop per 1 ml) showed values similar to those observed in the groups injected twice with saline (1.25 ± 0.21 vs 1.19 ± 0.16 (Mann Whitney U = 24, $p \leqslant 0.21$, NS). Table 3 shows the values for latency to burying and the mean number of electric shocks received (H = 2.499, df = 4, $p \leqslant 0.64$, NS and H = 7.743, df = 4, $p \leqslant 0.10$, NS, respectively). No changes

TABLE 2

THE EFFECT OF NALOXONE ON THE IP INDUCED CHANGES IN LATENCY OF BURYING AND THE NUMBER OF SHOCKS RECEIVED IN GROUPS DESCRIBED IN EXPERIMENT 2

| Groups | *n* | Latency of DB (min) | No. of Shocks |
|---|---|---|---|
| Control handled | 7 | 0.90 ± 0.42 | 2.42 ± 0.36 |
| 1.5 min after IP | 8 | 1.13 ± 0.38 | 2.00 ± 0.46 |
| 1.5 min × 2 (13.5)* | 8 | 0.64 ± 0.18 | 2.50 ± 0.32 |
| Naloxone control | 7 | 0.58 ± 0.09 | 2.14 ± 0.26 |
| Naloxone experimental | 7 | 1.21 ± 0.38 | 1.87 ± 0.26 |

*Control saline group injected twice (0 and 13.5 min) and tested for defensive burying 1.5 min after the second injection.

TABLE 3

THE EFFECT OF FLUMAZENIL ON LATENCY OF DB AND IN THE NUMBER OF RECEIVED SHOCKS IN ANIMAL GROUPS STUDIED IN EXPERIMENT 3

| Groups | *n* | Latency of DB (min) | No. of Shocks |
|---|---|---|---|
| Control handled | 7 | 0.90 ± 0.42 | 2.42 ± 0.36 |
| 1.5 min after IP | 8 | 1.13 ± 0.38 | 2.00 ± 0.46 |
| 1.5 min × 2 (28.5 min)* | 9 | 1.16 ± 0.24 | 2.54 ±0.44 |
| Flumazenil control | 8 | 1.04 ± 0.21 | 1.50 ± 0.26 |
| Flumazenil experimental | 7 | 1.00 ± 0.20 | 2.00 ± 0.21 |

*Control saline group injected twice (0 and 28.5 min) tested for DB 1.5 min after the second injection.

in the height of the bedding material were found (data not shown).

*Experiment 4: Effect of Naloxone on Defensive Burying 3 min After the IP Saline Injection*

Figure 4 shows the action of naloxone (1 mg/kg, IP) on defensive burying. The group injected twice with saline showed reduced DB, similarly to the animals injected once (0.49 ± 0.08 and 0.72 ± 0.14), whereas the group injected once with naloxone showed defensive burying times similar to those observed in control animals (1.32 ± 0.24 vs 1.32 ± 0.24). The group injected with naloxone at time 0 blocked the reduction in anxiety induced by the second saline solution injection when compared with the control handled non-injected group (1.32 ± 0.24 vs 1.38 ± 0.16, respectively). The Kruskal Wallis ANOVA test yielded the following values H = 12.630, df = 4, $p$ = 0.013. No significant changes were found in either the burying behavior latency or the number of shocks received (H = 4.361, df = 4, $p \leq 0.35$, NS and H = 4.518, df = 4, $p \leq 0.34$, NS, respectively, Table 4). No changes in the height of the bedding material were found (data not shown).

*Experiment 5: Effect of Flumazenil on Defensive Burying 3 min After the IP Saline Injection*

Figure 5 shows the action of flumazenil (5 mg/kg IP) in defensive burying, 3 min after the injection of saline. The group injected twice with saline exhibited reduced defensive burying levels similar to those shown by animals injected once

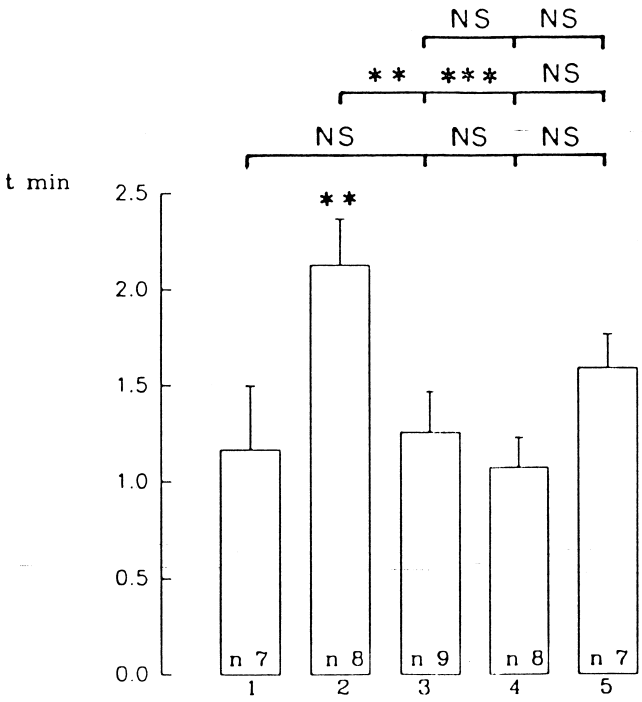


FIG. 3. Effect of flumazenil on the actions elicited by IP at 1.5 min of interval. Bars represent the mean time of DB ± SE of DB in the following groups: (1) control; (2) group tested for DB 1.5 after injection; (3) double injected control groups; (4) flumazenil control; and (5) flumazenil experimental group. Mann Whitney U test, NS: non significant; ***$p \leq 0.001$; **$p \leq 0.02$.

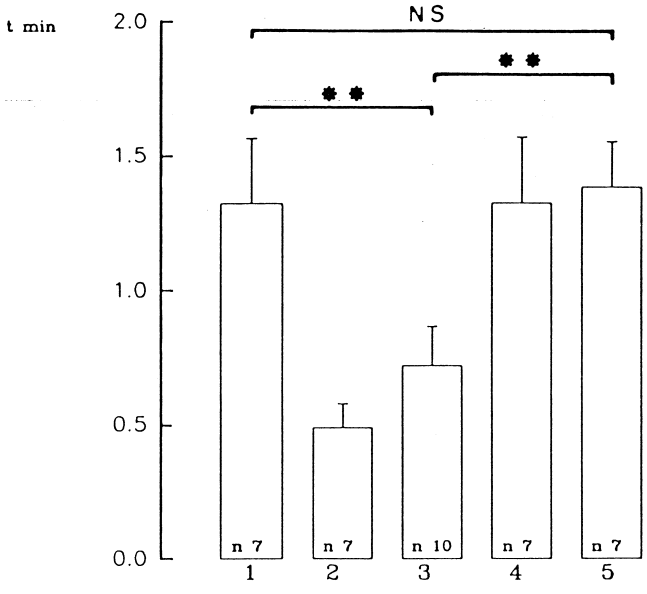


FIG. 4. Effect of naloxone on the decrease in burying behavior observed 3 min after the saline injection. Bars represent the mean time ± SE of burying behavior in the following groups: (1) control group; (2) group tested for anxiety 3 min after injection; (3) double saline injected group; (4) naloxone control group; and (5) naloxone injected experimental group. Mann Whitney U test, NS: non significant; **$p \leq 0.01$.

TABLE 4

THE EFFECT OF NALOXONE ON THE IP ELICITED ACTIONS ON LATENCY TO DB AND IN THE NUMBERS OF RECEIVED ELECTRIC SHOCKS IN ANIMALS STUDIED IN EXPERIMENT 4

| Groups | *n* | Latency of DB (min) | No. of Shocks |
|---|---|---|---|
| Control handled | 7 | 1.09 ± 0.21 | 1.42 ± 0.57 |
| 3 min | 7 | 0.89 ± 0.21 | 2.00 ± 0.53 |
| 3 × 2 (13.5 min)* | 10 | 0.89 ± 0.12 | 3.10 ± 0.48 |
| Naloxone control | 7 | 0.58 ± 0.09 | 2.14 ± 0.26 |
| Naloxone experimental | 7 | 1.39 ± 0.57 | 2.42 ± 0.36 |

*Control saline group injected twice (0 and 12 min) tested for DB 3 min after the second injection.

TABLE 5

THE EFFECT SHOWS THE ACTION OF FLUMAZENIL ON THE ACTIONS OF IP ON LATENCY OF BURYING BEHAVIOR AND IN THE NUMBER OF ELECTRIC SHOCKS IN ANIMALS STUDIED IN EXPERIMENT 5

| Groups | *n* | Latency of DB (min) | No. of Shocks |
|---|---|---|---|
| Control handled | 7 | 1.09 ± 0.21 | 2.42 ± 0.57 |
| 3 min after IP | 7 | 0.89 ± 0.21 | 2.00 ± 0.53 |
| 3 min × 2 (27 min)* | 11 | 0.87 ± 0.14 | 2.30 ± 0.39 |
| Flumazenil control | 8 | 1.04 ± 0.21 | 1.50 ± 0.26 |
| Flumazenil experimental | 7 | 0.59 ± 0.14 | 1.57 ± 0.29 |

*Control saline group injected twice (0 and 27 min) tested for DB 3 min after the second injection.

and tested 3 min after (0.49 ± 0.08 vs 0.60 ± 0.12, respectively). Flumazenil (5 mg/kg) failed to induce any changes in defensive burying (1.07 ± 0.15), whereas animals injected twice (flumazenil at 0 time and saline 27 min later) were able to revert the injection-induced reduction in burying behavior when com pared with the control handled non-injected group (1.32 ± 0.24 vs 1.38 ± 0.19, respectively). The Kruskal Wallis ANOVA test (H = 16.164, df = 4, $p \leq 0.002$) was significant. A control group injected with Tween 80 (1 drop per 1 ml) at time 0 showed no differences when compared with the control group injected saline twice (0.60 ± 12 vs 0.70 ± 0.14; Mann Whitney U = 30.5 $p \leq 0.23$ NS). Table 5 shows the values for latency of burying behavior and the mean of electric shocks received by different groups in the present experiment (H = 4.489, df = 4, $p \leq 0.34$, NS and H = 3.175, df = 4, $p \leq 0.52$, NS, respectively). No changes in the height of the bedding material were found (data not shown).

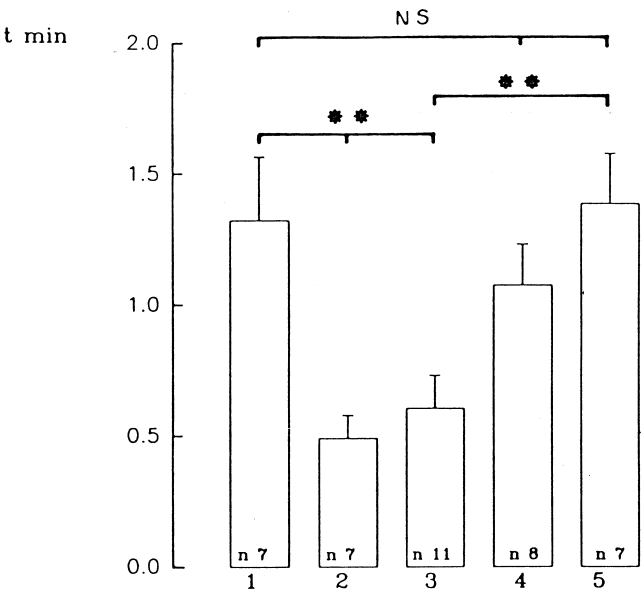


FIG. 5. Effect of flumazenil (5 mg/kg) on the decrease in anxiety induced by the injection. Bars represent the mean time ± SE of defensive burying for the following groups: (1) Control group; (2) group tested for anxiety 3 min after injection; (3) double saline injected group; (4) flumazenil injected control group; and (5) flumazenil injected experimental group. Mann Whitney U test, NS: nonsignificant; **$p \leq 0.01$.

*Experiment 6: The Temporal Course Effect of the IP Saline Injection on the Elevated Plus-Maze*

Figure 6 shows the temporal course action of the IP saline injection on the elevated plus-maze. The statistical analysis revealed no significant differences (Kruskal Wallis ANOVA H = 9.661, df, 6, $p \leq 0.13$ for open arms time and entries H = 8.487, df = 6, $p \leq 0.20$). The values of 1.5 and 3 min groups were corrected by ignoring the extreme values which did not alter the statistical lack of significance. Additionally, the paired Mann Whitney U test for control vs 1.5 min groups showed significant differences for the open arms times (U = 3, $p \leq 0.002$), while no such differences were found in the comparison between control vs the 3 min group (U = 16.5 NS). The 1.5 and 3 min group showed significant differences when compared among themselves (Mann Whitney U = 1 $p \leq 0.001$). The number of entries analyzed by this test revealed no significance both for 1.5 and 3 min with control values (U = 12.5 and U = 19 for groups tested at 10 and 15 min after injection (Mann Whitney U = 9.5, $p \leq 0.05$ and U = 8, $p \leq 0.05$, respectively; Fig. 6B).

## DISCUSSION

The main changes observed in the present work were increased DB levels 1.5 min and decreased burying behavior 3 min after IP saline injection (Fig. 1). The temporal course actions of the IP injection on defensive burying reveals no changes at 5, 10, 15, and 30 min after injection (Fig. 1). The analysis of double injected animals, and tested 1.5 min afterwards, revealed that this procedure failed to induce facilitated DB levels (Exps. 2, 3), as was observed in the single injected groups (Fig. 1). The administration of naloxone (1 mg/kg) blocked the reduction in DB observed in twice injected animals (Fig. 2), while flumazenil (5 mg/kg) was unable to revert the burying behavior (Fig. 3).

The reduction in DB could still be observed when animals were injected IP twice and tested for aversive response 3 min after injection (Figs. 4, 5). A blocking effect of both naloxone and flumazenil on the reduction in DB observed 3 min after the injection can be seen (Figs. 4, 5). A partial effect of IP injection in the elevated plus-maze was observed i.e., a decrease in the time spent in the open arms sections in animals tested 1.5 min after, and an increase in those individuals studied 3 min after saline injection, since the ANOVA test failed

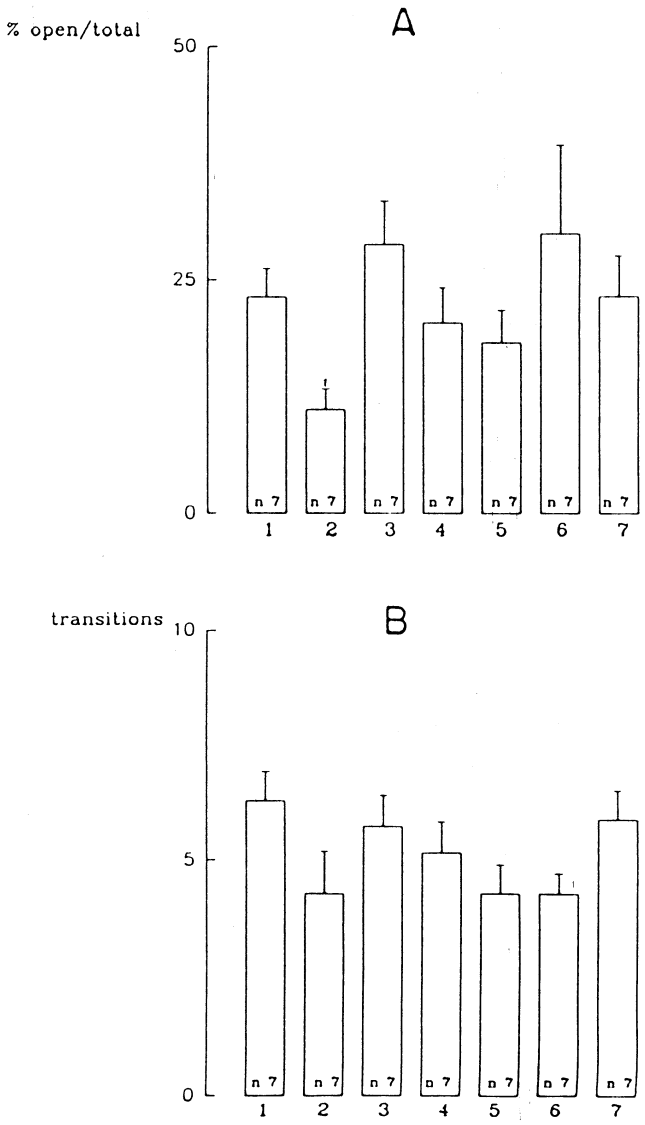


FIG. 6. Effect of IP saline injection on the elevated plus-maze for the followings groups: (1) control; (2) 1.5; (3) 3; (4) 5; (5) 10; (6) 15; and (6) 30 min after the IP injection. Panel A bars represent the mean time in % ± SE spent in the open arms sections for groups described above. Panel B. Bars represent the mean of the number of entries for groups described above. Mann Whitney U test, NS: non significant.

to show significant differences among groups (Fig. 6, A). However, when compared each of these groups independently, significant differences were found (see Results section; Fig. 6). The changes observed in the number of transitions from one closed arm to another, revealed a suggestive tendency between 1.5 and 3 min groups (Fig. 6 panel B), while significance was only found in late studied groups (10 and 15 min after IP injection for independent groups; see Results section), but not when the ANOVA test was used.

The temporal course action of IP injection on DB revealed rapid and transient effects in the animal's emotional status. The timing after the IP injection appears to be crucial, since the assessment for the aversive response early after the IP injection (1.5 min) facilitates the expression of DB, while a short interval later, DB expression is inhibited (Fig. 1). Several reports have described the induction of stress by delivering a nociceptive stimulus to the animal (6,9,23,60). Frequently, an electric shock causing a conflict between a positive reinforcer and the shock (23), during ambulatory activity (6) or water drinking (60) has been used. Recently, it has been reported that one single foot shock is able to reduce light dark transitions, an effect considered to reflect increased anxiety. It is interesting to note that the inverse agoinst to the benzodiazepine receptor, FG 71 42, induces a reduction in dark light transition. This finding suggests that the anxiogenic effect of the shock is mediated via the action of negative modulators of the benzodiazepine receptor (9,15). This evidence, showing the stressful nature of nociceptive stimuli, agrees with the evidence obtained in the present work, showing that animals tested 1.5 min after injection exhibited increased DB levels (Fig. 1). The fact that handling without injection, was unable to induce changes in DB, supports the idea that puncturing might be the critical element inducing changes in aversive responses. Although, previous reports have referred to the stressful character of handling (52,54), we did not observe any effect in DB. This is probably related to the fact that the time of handling in our work was very short, 32 sec, which was the mean time required for the injection (Fig. 1). In previous reports, the animals were usually handled far longer than the time used in our experiment (52,54).

Recently, our research group reported on the bimodal fluctuations in DB (increased-decreased), after exposure to the social interaction paradigm (47) and water drinking in an enforced water drinking design in rats (46). However, changes observed in those reports were less rapid compared with present findings (Fig. 1). These differences in time to show transient DB changes could be related to the nature of stressful manipulations used in those studies (46,47). The transient profile of modifications in DB (46,47) and actions of IP reported in the present work (Fig. 1), might confer the animal a putative long adaptive behavioral mechanism to cope with sequences of stressful events.

It is interesting to note that twice injected animals (Figs. 2, 3), tested for burying behavior 1.5 min after IP, showed a diminished DB level compared with single injected animals (Fig. 1). This effect supports the notion that the double injection elicits a protective action against facilitated DB (Fig. 2, 3). The fact that animals tested 15 and 30 min after one single injection (Fig. 1) show basal levels of DB, supports the idea that the second injection was performed in animals that started, in terms of the DB, approximately, from the basal values. The results obtained in experiments 2 and 3 suggest that the putative mechanism mediating changes in DB were partially activated, since the second IP injection induces an opposite effect to that observed in a single injected group. The activation of a rapid protecting mechanism lasting from 15 to 30 min after the first injection can be suggested (Figs. 2, 3). The study of DB in twice injected groups shows that burying behavior slowly returns back to facilitated values, increasing with time from the first injection raise (Fig. 2, 3). This fact suggests that the interval between stressful events is an important cue for the activation, or not, of an habituation mechanism. Probably a longer interval between injections, like 45 min, or on the contrary a shorter period, less than 3 min between injections, could lead to increased DB levels. This might represent the timing the protecting mechanism lasts.

The action of naloxone, in twice injected animals, tested 1.5 after the injection, supports the idea on the opiate nature of inhibited DB observed in this experiment (Fig. 2). On the

other, hand the inability of flumazenil to block this reduction indicates that the benzodiazepine receptor does not participate in its mediation (Fig. 3). Some evidence sustains the notion that repeated IP injection might act through a rapid habituation phenomenon, attenuating the expression of DB in double injected animals. This idea could be supported by reports that one single electroconvulsive shock is able to enhance the synthesis of enkephalines (62) and that repeated hot plate tests mask behavioral responses (19), an effect blocked by naloxone administration (42,43). The masking effect of repeated IP injections on DB could be related to the activation of the opiate system, evidenced by the blocking effect of naloxone (Fig. 2). One could suppose that the puncturing manipulation elicits the activation of a peptide antinociceptive mechanism, being responsible for decreased DB values; and that naloxone blocks the reduction in DB by impeding such a putative analgesic effect. However, the fact that no differences in the number of received shocks observed among groups in experiment 2, raises the possibility that a peptide system, not linked with nociceptive, but a stress related mechanism, could be responsible of a rapid increased-decreased DB in values observed in the present work.

The fact that both the opioid antagonist naloxone and the benzodiazepine receptor antagonist flumazenil, were able to revert the reduction in DB of the IP injection when animals were studied 3 min after (Figs. 4, 5), suggests that the opioid peptide and benzodiazepine receptors interact in the mediation of such an action. It has been reported that flumazenil, the selective Bz antagonist (27), may specifically revert the anxiolytic effect of various benzodiazepines without exhibiting intrinsic actions (38,39,59,53). In pharmacological approaches it has been proposed to use the high affinity of flumazenil with the Bz receptor (27) as a tool for discriminating the action of drugs through the GABA-Bz receptor complex. From this point of view, the fact that flumazenil at 5 mg/kg is able to block the reduction in anxiety induced by IP injection, without exhibiting intrinsic actions (Fig. 5), supports the participation of the benzodiazepine receptor in the reduction in DB occurring 3 min after the IP injection. Moreover, the action of flumazenil appears to favor the idea that the reduction of defensive burying in animals tested 3 min after IP injection is mediated via the activation of the benzodiazepine receptor, probably by the activation of putative endogenous ligands. It is interesting to remark that the transient activation occurred rapidly after the animals exhibited the opposite behavioral responses, increased anxiety 1.5 min after the injection. These two opposite actions, increased-decreased DB values, reveal a fast type of regulation, perhaps achieved by the interaction of opioid and benzodiazepine receptors. Recently, we have found that animals first tested on the hot plate nociceptive paradigm and immediately afterwards in the defensive burying anxiety model, exhibited diminished levels of defensive burying (unpublished data). These results seem to agree with the effects obtained in the present work (Fig. 1), where a nociceptive stimulus, IP induced rapid changes in DB levels, while no evidence on hyper or hyponociception were obtained (Tables 1–5).

The possibility of the involvement of opiates in the pharmacological effect of benzodiazepines is suggested by the changes in opioid peptide concentrations brought about by diazepam administration (16,17,61). Additionally, it has been reported that deprivation of natural reinforcers, such as food or water, results in stress increased states causing alterations in opioid binding (5,28,51,58). All this evidence supports the idea on the participation of opioid systems in stress responses, which might explain why naloxone returned, to increased DB levels (Figs. 2, 4) induced by the IP injection. On the other hand, it should be remarked that morphine has been used as a control for possible drug effects on anxiety in the defensive burying model and no reduction in DB was observed, providing experimental support for the assumption that the DB model reflects specific anxiety changes (56). It is important to note that the model is based on the aversion elicited by a very low intensity electric shock, raising doubts on whether the blocking effect induced by naloxone is associated with a diminished nociceptive threshold (Figs. 2, 4). Thus, the fact that morphine was ineffective to reduce DB (56), and the evidence presented in experiments 2 and 4, showing that naloxone lacks an intrinsic effect on DB (Figs. 2, 4), support the notion that the opioid system is participating in the mediation of increase (Figs. 1, 2) and decrease (Figs. 1, 4, 5) in DB via a non-nociceptive mechanism.

The analysis of the IP injection on the elevated plus-maze test revealed a partial but suggestive effect on the time the animals spent on the open arm section (Fig. 6A), and the number of transitions from one closed arm to another (Fig. 6B). Although the elevated plus-maze test has been validated as a useful paradigm for preclinical drug screening (13,36), we found, however, that in the present design the EPM represents a less sensitive paradigm.

In conclusion, IP saline injection induced transient changes in DB and partial actions in the EPM paradigm. The above presented evidence supports the notion that both opioid and benzodiazepine neurotransmitter systems participate in the mediation of changes in DB induced by IP saline injection. However, more experiments should be undertaken to verify some of the above proposed hypotheses, as well as to elucidate the possible participation of other neurotransmitter systems such as serotonergic, dopaminergic and norepinephrine on the IP injection elicited changes.

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