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Chronic Exposure to Flumazenil: Anxiolytic Effect and Increased Exploratory Behavior

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URBANCIC, M., M. A. GADEK AND T. J. MARCZYNSKI. *Chronic exposure to flumazenil: Anxiolytic effect and increased exploratory behavior*. PHARMACOL BIOCHEM BEHAV **35**(3) 503–509, 1990.—The aim of the present study was to define the behavioral correlates of chronic exposure of adult rats to flumazenil (4 mg/kg/day × 21 days in drinking water). In the holeboard test, performed on day 13 of drug treatment, the animals showed a significantly greater interest for the holes under which objects were placed than for the holes without objects ($p<0.03$), while there was no such difference in the control group. In the plus-maze test, the flumazenil-treated animals spent significantly more time on open arms and left less fecal boluses than the controls when tested in the third week of treatment and 24 hours after flumazenil withdrawal. In the drinking-punishment test, conducted on days 3, 6 and 10 after drug withdrawal, the drug-exposed animals, following shock experience, did not significantly alter their unpunished drinking in subsequent trials, while the control rats significantly reduced ($p<0.003$) their unpunished drinking. Also, the punished drinking revealed a significant "anticonflict" effect of prior exposure to flumazenil ($p<0.006$) which was still observed 6 days after drug withdrawal. There were no group differences in the home-cage food and water consumption during flumazenil treatment; also, the drug treatment had no effect on nociceptive threshold. In summary, chronic treatment with a benzodiazepine receptor antagonist, flumazenil, increased exploratory activity and had a lasting anxiolytic effect.

Flumazenil Ro 15-1788 Benzodiazepine receptors Anxiolytic effect Exploratory activity

IN 1981, an imidazodiazepine, Ro 15-1788, was discovered that specifically antagonized the effect of benzodiazepines (BDZs) in a variety of biochemical, electrophysiological and behavioral tests (19,28). Although originally considered as a "neutral" BDZ antagonist (2), evidence is accumulating that Ro 15-1788 (flumazenil) is not devoid of intrinsic pharmacological actions. Flumazenil has been shown to have behavioral actions of its own which can be classified as either agonist or inverse agonist, depending upon the test condition and the dose used. In rats, acute administration of flumazenil has anxiogenic effects in the social interaction test in doses of 10 mg/kg, IP, but not in doses of 4 and 20 mg/kg, IP (10,12), in the punished drinking test, if injected in doses of 20 and 30 mg/kg, IP (13), and in a test of food (8 mg/kg, IP) and water consumption (10 mg/kg, IP) in novel environment (13,18).

Some of the animal tests of anxiety have failed to detect any action of flumazenil; for instance, the elevated "plus maze" [10–20 mg/kg and 4 mg/kg, IP (1,32)], and the punished drinking test, when injected in doses of 2 mg/kg IV (6,27) or in doses of 25 mg/kg, IP (36), and in doses of up to 100 mg/kg, PO (3). On the other hand, flumazenil (4–20 mg/kg, IP) was found to increase exploratory behavior in the holeboard test, without affecting locomotor activity (11), and to exhibit a weak antiaversive action (35 mg/kg, IP) as measured by the latency of escape reaction to electrical stimulation of the periaqueductal grey matter (22). The latter actions of flumazenil could be classified as BDZ agonist-like. In human subjects, flumazenil also showed BDZ-like agonist actions in several psychophysiological tests, such as EEG spectra, blood pressure, tremor, reaction time task, etc. (17). Generally, in rodents, relatively low single doses of flumazenil (4–10 mg/kg) appear to have anxiogenic effects, while higher doses (20–50 mg/kg) seem to have weak diazepam-like anxiolytic effects (7,30).

Little is known about the effects of chronic administration of flumazenil. In rats, perinatal exposure to flumazenil, 3 mg/kg for 3 weeks administered in drinking water to pregnant and subsequently lactating dams during ontogeny of BDZ receptors, had a lasting effect in adult, 5 months old offspring, as reflected by increased BDZ receptor numbers in the hippocampal formation and the associated more efficient and "fearless" goal-directed behavior, compared to offspring perinatally exposed to diazepam or the drug vehicle (23,24). In contrast, a higher dose of flumazenil (20 mg/kg SC), administered to pregnant rats during the last week of gestation, resulted in a decrease of neocortical BDZ binding and a lower seizure threshold, as measured in 18 days old offspring (14).

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Chronic exposure of adult rats to flumazenil (4 mg/kg for 14 days in drinking water) increased the number of BDZ and beta-carboline binding sites (26). Using the same 2-week treatment regimen, we have confirmed the above observations and, in addition, we have found a significant decrease in GABA facilitation of flunitrazepam binding to neocortical membranes of flumazenil-exposed rats, a change that was still present 72 hours after drug withdrawal (35).

Prompted by the above receptor studies, the aim of our present investigation was to evaluate the behavioral correlates of chronic 21-day exposure of adult rats to flumazenil, by focusing on the time period between day 13 through day 21 of daily drug administration, and including the time period of 10 days after drug withdrawal. Toward this end, the following behavioral tests were used: 1) those that are believed to measure anxiety, such as the elevated "plus-maze" test (31) and the drinking-punishment test (37); 2) the holeboard test that measures both the exploratory and the locomotor activity (9); 3) tests that measure the threshold to painful stimuli: the tail-flick test and the tail-shock vocalization test; and, finally, 4) we evaluated the food and water intake and the changes in body weight of the chronically treated animals.

METHOD

Animals and Drug Administration

Male Sprague-Dawley rats (Bio-Lab Corp., Saint Paul, MN), weighing 270–290 g at the beginning of the study, were used (for control group $n = 12$, for flumazenil-treated group $n = 11$); they were singly housed in an air-conditioned room with a 14-hr light/10-hr dark cycle (lights on at 0600 hr) and were allowed free access to food (Purina Chow) and water. After the average daily water consumption during a 7-day period has been ascertained, flumazenil (Ro 15-1788, 4 mg/kg/day) was dissolved in ethylene glycol (5 ml per 1000 ml of tap water) and administered in drinking water for 3 weeks. The drug was generously provided by Dr. Peter Sorter from the Hoffmann-La Roche Co. (Nutley, NJ). The control group received a comparable volume of the drug vehicle in drinking water. The rats tended to drink less per kg of body weight parallel with an increase in their body weight, e.g., the mean volume of the consumed water for both groups on day 1 equaled 131.3 ml/kg, while on the last day 21 of treatment this value equaled 97.0 ml/kg. The volume of consumed water was measured every 24 hours and any significant change in water consumption was compensated by adjusting the drug concentration to optimize the intended dose of 4 mg/kg/day. The average daily dose of flumazenil equaled 4.0 ± 0.2 SD mg/kg.

Apparatus and Procedures

All experiments were carried out between 0800 and 1200 a.m. After each trial, fecal boluses were counted and removed from the test arena and the floor was thoroughly cleaned. The behavior in the plus-maze was recorded on video tape and later analyzed by two investigators, one of whom had no knowledge of the drug state of the animals.

Holeboard test. The apparatus was a wooden box $60 \times 60 \times 36$ cm, with four equally spaced holes in the floor, each 3.5 cm in diameter; various objects were placed under two of the holes (9). The level of illumination was 30 scotopic lux. The infrared photocells, placed under each hole, monitored the number of the animal's head-dips and the time spent head-dipping, while the photocells placed in the walls measured the locomotor activity. Each rat was tested for 5 min on day 13 of chronic drug treatment.

Elevated plus-maze test. The apparatus (31) consisted of four horizontal wooden arms: two opposite open arms (50×11 cm), and two opposite enclosed arms ($50 \times 11 \times 40$ cm), connected by a central platform (11×11 cm). The maze was elevated 50 cm above the floor. In a variant of this test, the access to two enclosed arms was blocked allowing the animal either to enter the open arms or to stay in the partially enclosed center platform; the elevation of the maze was reduced from 50 to 25 cm above the floor. In both variants of the test, the level of illumination was 22 and 30 scotopic lux for protected and open arms, respectively.

The first test was conducted on day 13 of drug or vehicle treatment, immediately after the animal was subjected to a 5-minute test in the holeboard box and, therefore, had time to habituate to handling and novel environment. The remaining tests in the plus-maze (from day 15 through 21 of drug treatment and 24 hours after drug withdrawal) were carried out without prior holeboard test, i.e., each animal was transferred directly from its home cage to the plus-maze. After the first six daily 7.5-min trials in a regular plus-maze, the animals received additional three daily 5-min tests in the plus-maze with the enclosed arms blocked. The repeated daily tests enabled us to assess the effect of increasing familiarity with a test arena on the animal's behavior. Also, closing the entrance to protected arms, followed by water deprivation, provided insight into a potential role of novel exogenous and endogenous stimuli on the animal's behavior. The number of entries and total time spent in each type of arms were scored. There is a convincing evidence that validates this simple test as a sensitive tool for measuring the exploratory behavior and the anxiolytic or anxiogenic action of drugs (31).

The drinking-punishment test. This test was performed in a clear Plexiglas cage, the same in which the rats were housed, but without bedding and equipped with a stainless steel floor. The metal drinking spout and the floor were connected to a constant current shock generator and to the drinkometer (Columbus International, Inc., Columbus, OH). Two days prior to the initiation of drug treatment and 24 hours after water deprivation, rats were tested for the latency to start drinking and number of unpunished licks per one minute. On the basis of these tests, the animals were divided into two comparable groups for flumazenil and vehicle treatment. On days 3, 6 and 10 after drug withdrawal, the 44-hr water-deprived animals were tested for 1 min of unpunished drinking, followed by a 5-min period of punished drinking. After every 20 licks, 0.35 mA current was delivered to the drinking tube. Measures were: the latency to the first lick, the number of licks in unpunished and punished periods, and the number of shocks received.

Tail-flick reflex latency. The rat's tail was placed under an intense light source and the latency for tail withdrawal was measured. The time between the onset of the light stimulus and the triggering of the photodetector was defined as the tail-flick latency. The test was carried out on day 6 after drug withdrawal.

Tail-shock vocalization test. Two aluminum foil bands were placed around the midportion of the rat's tail and alternating current, ranging from 25 to 60 mA, was applied to the tail for 0.1 sec [for details, see (4)]. The lowest current intensity at which the rat vocalized was used as the threshold for painful stimuli. The test was carried out on day 8 after drug withdrawal.

Statistics

The differences in behavioral scores within a group over daily trials and between two groups of animals over time during and after chronic administration of the drug or drug vehicle were ascertained using the BMDP software for one- or two-way analysis of variance (ANOVA) with drug treatment, or drug/vehicle withdrawal and days as factors, or ANOVA with repeated measures. If the distribution of the data met the requirements, the *t*-test

was used to compare two samples of data. If the data did not fulfill the assumptions of ANOVA, they were logtransformed. In instances of significant departures from normal distribution of the data or dependence of variances on the means, the nonparametric tests were used to ascertain the within-group differences over time (the Wilcoxon Matched Pairs Rank Sum test). The differences between two groups in the numbers fecal boluses left in the test arena were analyzed by chi-square test.

RESULTS

Holeboard Test

On day 13 of chronic treatment with flumazenil, there were no significant differences in the mean number of head-dips between the groups (control, 15.7 ± 1.8 SE; drug, 17.5 ± 3.1 SE) nor in the time spent head-dipping (control, 25.2 sec ± 3.3 SE; drug, 23.6 sec ± 2.8 SE). However, the flumazenil-treated animals showed a significantly greater interest for the holes were the objects were present than for the holes without objects, $F(1,20) = 5.5$, $p < 0.03$, whereas in the control group the time spent head-dipping at the holes with or without objects was not significantly different, $F(1,22) = 2.8$, $p = 0.11$.

Flumazenil had no effect on locomotor activity (control: 526 ± 40 SE; drug: 547 ± 36 SE).

Plus-Maze Test

The animals were tested daily, beginning on day 13 through day 21 of continuous flumazenil treatment (except day 14), and 24 hr after drug withdrawal and water deprivation (W1; abscissa; Fig. 1, top). Trial 1 was performed immediately after each animal spent 5 min in the holeboard box, while the subsequent trials were done without prior exposure to the holeboard, in order to see whether or not the change in the procedure (with or without acclimation in the holeboard) would have a differential effect on behavior of the control and the drug-treated group in the plus-maze test. The results showed that indeed this procedure disclosed significant differences between the flumazenil-treated group and the control group. In trial 1 that immediately followed the holeboard exposure (day 13 of drug treatment), there were no significant differences between the control and the drug-treated group in the mean percent time the animals spent on the open arms (ordinate of Fig. 1 top). However, in trial 2 conducted 48 hr later (day 15 of drug treatment) the control group showed a decrease in the mean percent time spent on open arms, as compared to trial 1 ($p < 0.02$, paired t-test) and this exploratory behavior continued to be significantly depressed (s) through trial 4 (day 17 of drug treatment; $p < 0.02$). Conversely, the behavior of the drug-treated rats was not significantly suppressed by the lack of prior exposure to the holeboard box, and on day 17 and 18 (trial 4 and 5, respectively), the percent time spent on open arms was significantly higher in the drug-treated rats, as compared to the control ($p < 0.05$ and $p < 0.03$, respectively; two-tailed t-test). A comparison of both groups over trials 2 through 6 showed a significantly increased exploratory behavior of flumazenil-treated rats [two-way ANOVA; $F(1,105) = 7.68$, $p = 0.007$]. On day 19 (trial 6), the control rats showed signs of habituation to the test apparatus and their time spent on open arms was not different from that of drug-treated animals and was also comparable to their postholeboard (trial 1) performance. Judging from the time course of exploratory behavior of either group, in the control group, the aversion to the open arms remained unchanged over time [trials 2 through 6; $F(4,44) = 1.8$, $p = 0.15$], while in the flumazenil-treated group, there was a significant increase in exploratory behavior over time, $F(4,40) = 15.31$, $p = 0.0016$.

In the next trial 7 (day 20 of drug treatment), the animals were confronted with a novel situation in the plus-maze design, since both entrances to the enclosed arms were blocked. This change disclosed group differences, since it triggered a stronger exploratory response in the drug-treated animals, relative to controls ($p < 0.04$; two-tailed t-test). On the last day 21 of drug treatment (trial 8), the difference between the two groups was not significant, but the next day (trial 9), i.e., 24 hr after drug withdrawal and water deprivation, the drug-exposed rats again spent significantly more time on the open arms than the controls ($p < 0.04$).

The mean number of fecal boluses the animals left after each test (ordinate of Fig. 1 bottom panel) reflected the group differences in the time course of habituation to the testing procedure: in the initial 3 trials, the 12 control rats in $3 \times 12 = 36$ individual trials left a total of 28 boluses, and the ratio of 28/36 was comparable to that for 11 drug-exposed rats—31/33, $\chi^2(1) = 0.13$, $p = 0.72$. However, in subsequent 5 daily trials, the sum total ratio of 73/60 in the control group was higher than the ratio of 14/55 for the drug group, $\chi^2(1) = 20.79$, $p < 10^{-5}$.

When comparing the time course of the daily defecation scores with the time course of the mean percent time the animals spent on open arms over the 8 sequential trials (Fig. 1, the bottom and the top panels, respectively), it became apparent that there was an inverse relationship between these two measures. Moreover, by plotting for each trial the mean numbers of fecal boluses left by the animals in the plus-maze versus the mean percent time the animals spent on open arms (Fig. 2), a significant negative linear regression was obtained ($r = -.64$; $n = 16$; $p = 0.007$).

In addition, the drug-treated animals, compared to the control group, spent significantly less time in the enclosed arms, when tested on day 17 and 18 of continuing treatment (trial 4 and 5; Fig. 3).

The increased exploratory activity of the flumazenil-treated animals was not only expressed by the increased percent time spent on open arms, but also by significantly higher number of total arm entries [$F(1,126) = 9.43$, $p < 0.003$, for trials 1 through 6]. Also, in trial 7, when the entrances to the enclosed arms were blocked, and in trial 9, following a 24-hr water deprivation and drug withdrawal, the drug-treated group made significantly more entries into the open arms than controls (Table 1).

Drinking-Punishment Test

Chronic 21-day exposure to flumazenil had a significant "anticonflict" effect on both unpunished and punished drinking (ordinate in Fig. 4, left and right, respectively) as tested on days 3, 6 and 10 following drug withdrawal (abscissa). The response magnitudes in the unpunished predrug trial and in trial on day 3 after drug/vehicle withdrawal, reflected a shock-naive state of the animals, and the group mean responses were virtually identical. However, the first shock the animals experienced on day 3 of vehicle withdrawal significantly reduced the unpunished drinking in the control group during the subsequent test ($p < 0.003$; Wilcoxon Matched Pairs Signed Rank test); the second shock experienced on day 6 of vehicle withdrawal further deepened this suppression on day 10. On the other hand, the unpunished drinking in the drug group, compared to the naive condition, was not significantly altered after shock experience (ns; $p = 0.57$ and $p = 0.17$, for day 6 and 10, respectively) and, on day 10 of drug withdrawal, there was a significant difference in unpunished drinking between the control and the drug-exposed group ($p < 0.006$; two-tailed t-test). The ANOVA also showed that in control group, the shock experience significantly reduced unpunished drinking, $F(2,30) = 14.6$, $p < 0.00004$, while in the drug-treated group, the unpunished drinking was not altered by shock experience, $F(2,27) = 0.98$, $p = 0.39$.

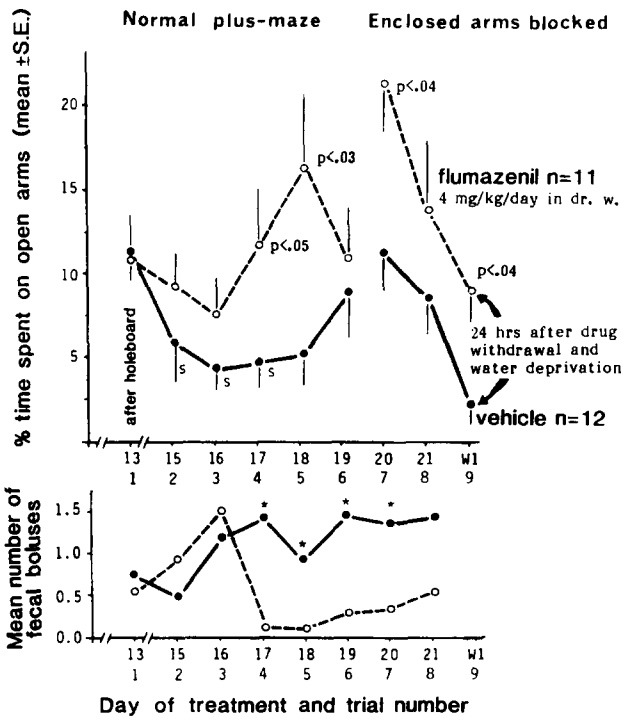

FIG. 1. (Top) Enhanced exploratory behavior of rats during chronic 21-day treatment with flumazenil (4 mg/kg/day in drinking water), as measured by the group mean percent time spent on open arms of the elevated plus-maze (ordinate). Rats were tested on day 13 and day 15 through 21 of drug or vehicle treatment, and 24 hours after drug or vehicle withdrawal (W1; abscissa). Trial 1 (day 13) was conducted immediately following a 5-min holeboard test which, to some extent, habituated the animals to handling and novel environment and, as a result, both groups showed comparable exploratory behavior. However, in subsequent trials without prior holeboard exposure, the control group was significantly (s; $p<0.02$; paired t-tests) more reluctant to explore the open arms than on the postholeboard trial, while the drug-treated group was not significantly inhibited, and on day 17 (trial 4) and day 18 (trial 5) the differences between the groups were significant ($p<0.05$ and $p<0.03$; two-tailed t-tests). After the entrance to the enclosed arms had been blocked (trial 7 through 9), the drug-treated group spent more time on open arms than the control group ($p<0.04$). In the last trial, conducted 24 hr after drug withdrawal and water deprivation, again a significant difference between the two groups emerged, the drug-exposed group showing more exploratory behavior than the control group ($p<0.04$). (Bottom) The mean numbers of fecal boluses (ordinate) the animals left in the plus-maze after each of 1 through 8 trials (abscissa). Note that the drug-treated group, in trials 4 through 8, almost ceased defecating and the difference in defecation score between the two groups was highly significant ($p<10^{-5}$).

Punished drinking (right panel of Fig. 4) that followed 1 min of unpunished drinking also revealed "anticonflict" effect of prior exposure to flumazenil, as tested in 5-min trials on day 3 and 6 after drug or vehicle withdrawal. The drug-exposed animals, on the average, received 10.9 ± 2.8 SE shocks and 19.3 ± 4.2 SE shocks on day 3 and 6, respectively, while the control animals received only 4.5 ± 0.8 SE and 6.8 ± 1.3 SE shocks, respectively, and these differences were significant ($p<0.04$ and $p<0.006$, respectively; two-tailed t-test).

The latency to the first lick was not affected by drug withdrawal, nor by the shock experience.

Tail-Flick Test and Tail-Shock Vocalization Test

The latency of the tail-flick reflex and nociceptive threshold, measured by the tail-shock vocalization test, were not altered in the rats treated with flumazenil, as compared to controls, on day 6 and 8 after drug withdrawal (Fig. 5).

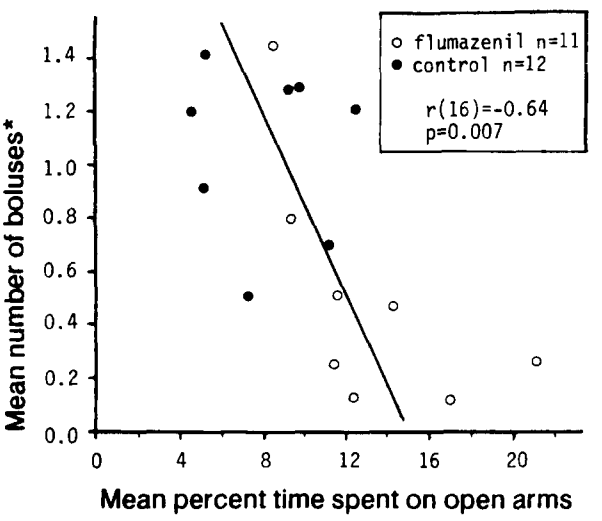

FIG. 2. Significant inverse relationship ($r = -.64$; $p = 0.007$) between the mean number of fecal boluses left by the control group and the drug-treated group after each of 8 trials in the plus-maze (ordinate) and the mean percent time each group spent on open arms of the plus-maze (abscissa). *Per each of 16 trials.

Food and Water Consumption

On the average, the water intake in the drug-treated group (112.8 ± 18.0 SD ml/kg/24 hr) over the time period of 21 days was not significantly different from the vehicle-treated group (119.8 ± 13.7 SD ml/kg/24 hr) [$F(1,16) = 1.64$, $p = 0.22$; ANOVA with repeated measures]. Also, over the same time period, the daily food consumption was not significantly different in both groups, $F(1,16) = 0.32$, $p = 0.58$; it equaled 15.4 ± 2.6 SD g/200 g body

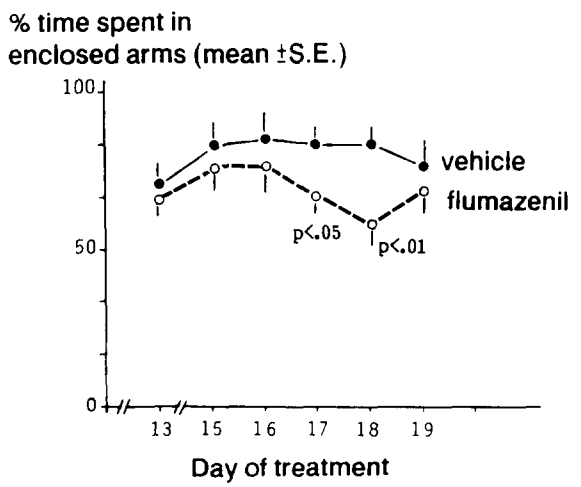

FIG. 3. Effect of chronic treatment with flumazenil on the mean percent time spent in the enclosed arms of the elevated plus-maze. p Values for two-tailed t-test.

TABLE 1

MEAN (±S.E.M.) NUMBER OF ENTRIES AND TIME SPENT ON TWO OPEN ARMS (5-MIN TEST)

Time of Testing	Control	Ro 15-1788 (4 mg/kg/day)
Day 20		
number	3.8 ± 0.8	6.0 ± 0.5*
time (sec)	36.2 ± 8.5	63.3 ± 7.7*
Day 21		
number	2.5 ± 0.7	3.4 ± 0.8
time (sec)	26.3 ± 8.8	42.2 ± 14.5
24-hr water deprivation		
number	0.7 ± 0.3	2.6 ± 0.8†
time (sec)	8.3 ± 3.4	28.0 ± 8.1*

*$p<0.05$, †$p<0.03$ for difference from control (Student's t-test).

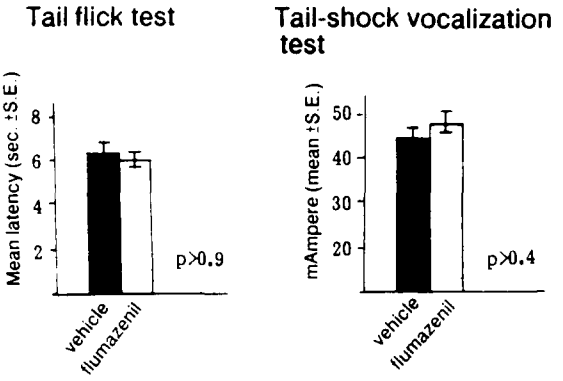

FIG. 5. Lack of antinociceptive effect of the chronic flumazenil treatment, measured on day 6 (tail-flick test) and day 8 (tail-shock vocalization test) after drug withdrawal, i.e., during the time when a significant "anticonflict" effect of the prior drug exposure was observed.

weight in the control group, and 15.0±1.6 SD g/200 g body weight in the drug group.

DISCUSSION

We have observed a moderate anxiolytic effect of chronic exposure to BDZ antagonist, flumazenil and, paradoxically, this effect was followed by an even more pronounced anxiolytic effect observed for several days after drug withdrawal. The following results indicate anxiolytic effects of chronic flumazenil treatment:

First, toward the end of the third week of flumazenil treatment and 24 hr after drug withdrawal the animals spent more time on open arms of the elevated plus-maze than the control group; such behavior was observed by other investigators following acute or chronic administration of BDZ agonist, such as chlordiazepoxide and diazepam (31), but not after acute treatment with flumazenil (32).

Second, in the plus-maze test, the defecation/urination scores, believed to reflect a temporary imbalance in the autonomic nervous system caused by emotional responses to novel environmental stimuli (34), were significantly decreased in flumazenil-exposed animals, as compared to controls. The fact that the exploratory behavior of the animals in the plus-maze was inversely

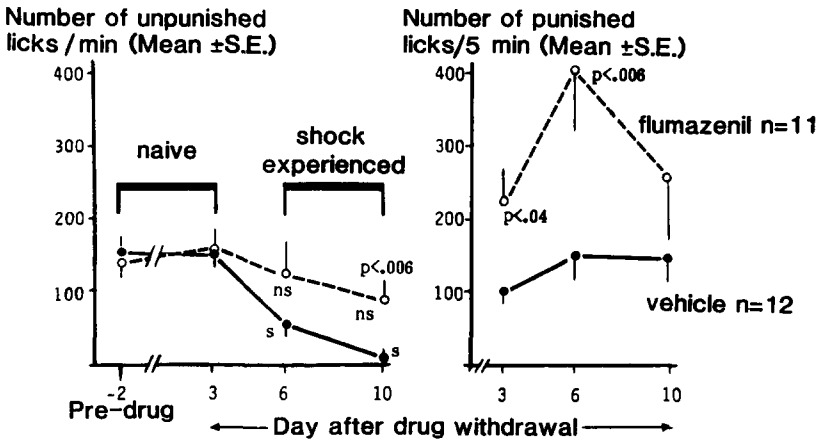

FIG. 4. The "anticonflict" effect of chronic exposure to flumazenil on the mean number of unpunished and punished licks (ordinate; left and right panel, respectively) plotted for days 3, 6 and 10 following drug withdrawal (abscissa). One min of unpunished drinking was followed by 5 min of punished drinking (0.35 mA shock after each 20 licks), except for the predrug test (2 days prior to initiation of the treatment). Each test was carried out after 44-hr water deprivation. In four trials of unpunished drinking (left panel), the condition of the animals can be divided into a shock-naive (predrug and day 3 after drug or vehicle withdrawal), and a shock-experienced condition (day 6 and 10 after drug or vehicle withdrawal). Note that, in the control group, shock experience significantly (s) inhibited unpunished drinking, compared to each of the two naive state trials ($p<0.003$; Wilcoxon test), while shock experience had no significant effect (ns; $p=0.14$) on the drug exposed group; on day 10 of drug withdrawal, there was a highly significant difference between two groups ($p<0.006$; two tailed t-test). Also, the punished drinking (right panel) was significantly enhanced on days 3 and 6 after drug withdrawal ($p<0.04$ and $p<0.006$, respectively; t-test), as compared to the control group.

correlated with the defecation scores indicate that the drug-exposed animals, compared to controls, were less emotional and their autonomic nervous system was more stable when challenged by novel environmental stimuli. Actually, in most instances, it was a challenge, environmental or endogenous, that unmasked the behavioral group differences: 1) the initial use of the holeboard box for acclimation and then abandoning this procedure; 2) blocking the entrances to the enclosed arms of the maze; and 3) water deprivation, each triggered significantly stronger exploratory reaction in the drug group.

The unaltered locomotor activity and the total time spent head-dipping in the holeboard test found by us during chronic flumazenil treatment, have also been described in rats chronically treated with the BDZ agonists, chlordiazepoxide and diazepam (31). Thus, one may be tempted to ascribe the above mentioned anxiolytic effects of chronic flumazenil to its weak BDZ agonist action. There is, however, a fundamental difference between the typical BDZ agonists and flumazenil: while the BDZ agonists, upon termination of chronic treatment, cause withdrawal symptoms that are indicative of emotional tension and anxiety (1, 20, 29), flumazenil withdrawal, in the present study, was characterized by unabated, if not potentiated, anxiolytic effect lasting at least 10 days after drug withdrawal as measured in the drinking-punishment test. Hence, we can conclude that the anxiolytic or "anticonflict" action of chronic flumazenil cannot be explained by its presumable weak BDZ agonist properties.

Three issues should be considered when searching for a plausible explanation of our observations: 1) the synthesis and the levels of a potential endogenous ligand(s) for BDZ receptors, 2) conformational changes within GABA/BDZ receptor complex, and 3) the influence of chronic flumazenil on other neurotransmitter and/or modulator systems.

Endogenous Ligand(s)

A polypeptide, labeled as a diazepam binding inhibitor (DBI), endowed with an anxiogenic, "proconflict," or "inverse-agonist"-like action, has been isolated from rat brain homogenates (16). It was suggested that DBI and/or its biologically active fragments could be stored and protected from further degradation in specific cellular compartments (8). Also, polypeptides from the bovine and human brain have been isolated that display a significant sequence homology with DBI (25). It is thus conceivable that a chronic occupation of BDZ recognition sites by flumazenil prevents the physiological action of DBI, if this peptide and/or its active fragments are released when the animal is challenged by "intimidating" and/or stressful stimuli. In such a condition, flumazenil can be expected to have a stabilizing effect on emotional responses normally triggered by DBI, without having any significant effects of its own. Such a scenario of flumazenil action is compatible with our results, since, as mentioned above, differences between the flumazenil-treated animals and the controls did indeed emerge in response to novel and challenging stimuli. However, the apparent lack of anxiogenic effect of flumazenil withdrawal in the plus-maze test and the drinking-punishment test does not support the theory of flumazenil "protection" of BDZ receptors from DBI or its active fragments.

On the other hand, an endogenous BDZ agonist has been recently purified from bovine brain, and benzodiazepine-like immunoreactivity was detected in human brain from subjects who have not been exposed to BDZs (33). The purified substance was identified as the N-desmethyldiazepam, known as a common metabolite of BDZs, whose elimination half-life ranges between 50–100 hr and has a tendency to accumulate in the body after prolonged BDZ treatment (15). A chronic competition of flumazenil with the endogenous BDZ-like receptor ligand during the treatment could be expected to cause effects opposite to those observed in the present study. However, accumulation of a BDZ-like endogenous ligand during flumazenil treatment could, in theory, overcome the receptor blockade by flumazenil and displace the antagonist from BDZ receptors, thus producing an anxiolytic effect. An increased synthesis and release of a BDZ-like ligand could account for the anticonflict effects following drug withdrawal.

Conformation Changes in BDZ/GABA Receptor Complex

The mechanism of behavioral changes reported here may be more complex than the consequences of a competition of flumazenil for BDZ receptors. It has been suggested that flumazenil, in vitro, is especially efficient in shifting the BDZ receptor to the high-affinity conformation and that such an activity would mask the effects of agents known to enhance binding of benzodiazepine agonists, such as GABA, NaCl or pentobarbital (5). We have found a significant decrease in GABA enhancement of [^{3}H] flunitrazepam binding to rat neocortical BDZ receptors that was still present 3 days following a two-week flumazenil treatment (35), and this finding may reflect a conformation change in the BDZ/GABA receptor complex and altered coupling between the GABA and the BDZ recognition sites caused by flumazenil.

The upregulation of the central BDZ receptor density found after chronic flumazenil treatment (26) does not persist after drug withdrawal: it may last for up to 24 and 48 hr in the hippocampus and neocortex, respectively, the affinity remaining unchanged (35). Thus, in the present study, the significant anxiolytic effect of flumazenil observed 10 days after flumazenil withdrawal cannot be explained by alteration in the number nor affinity of BDZ receptors.

Possible Interaction With the Cholinergic System

As already mentioned, the increased exploratory behavior of the flumazenil-treated animals in the plus-maze test emerged whenever a change in the testing procedure and/or environment was introduced. This tendency may be explained by faster habituation and increased vigilance in the drug-treated animals. Such an interpretation is compatible with the suggestion made by other investigators (21) that flumazenil enhances vigilance and memory by antagonizing the suppressant influence on the cholinergic system normally exerted by the BDZ-like endogenous ligand(s) acting via the GABA/BDZ receptor complex.

In conclusion, on the basis of our current knowledge of the GABA/BDZ receptor complex, none of the above discussed mechanisms, if considered alone, can fully explain the anxiolytic effects of chronic flumazenil treatment and the apparent absence of withdrawal symptoms.

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