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Behavioral effects of flumazenil in the social conflict test in mice

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Abstract *Rationale:* Flumazenil, a competitive antagonist of benzodiazepine receptors (BZR), has been used as a probe to detect effects of putative endogenous ligands for BZR in anxiety. Flumazenil is renowned for its highly inconsistent behavioral effects. *Objective:* To ascertain effects of flumazenil in the social conflict test in mice, which provides complex measures for prediction of anxiolytic and anxiogenic activity of drugs in behaviorally different groups of animals. *Methods:* Singly housed male mice treated with flumazenil (5, 20 or 80 mg/kg i.p.) or vehicle were paired with untreated non-aggressive group-housed male mice in a novel cage. Behavior was analyzed from video tapes of the social interactions in three populations of mice: timid ($n=21$), aggressive ($n=11$), and sociable ($n=7$). Levels of γ -aminobutyric acid (GABA) were measured in vivo in the prefrontal cortex. *Results:* Flumazenil reduced timid (defensive-escape) and increased locomotor activities in timid mice. The drug reduced aggressive and increased sociable (social investigation) activities in aggressive mice. These behavioral changes were produced at the lowest dose of flumazenil tested (5 mg/kg) and were not increased further by higher doses of the drug (20 mg/kg or 80 mg/kg). A tendency to increased timidity was found after flumazenil in sociable mice. Concentrations of GABA were markedly higher in the prefrontal cortex of sociable mice than in timid or aggressive mice. *Conclusions:* Flumazenil produced moderate anxiolytic-like behavioural changes and a slight anxiogenic-like effect. The present data might be reflecting antagonism of corresponding endogenous BZR ligands. However, these putative ligands seem to exert only modest modulatory influence.

Keywords Flumazenil · Defensive behavior · Anxiety · Benzodiazepine receptor · Endozepines · Endogenous ligands · Individual differences · Aggressive behavior · GABA

Introduction

Flumazenil is a competitive antagonist of benzodiazepine (BDZ) receptors (BZR, also termed the BDZ binding sites; Barnard et al. 1998), which are present as allosteric modulatory sites at the γ -aminobutyric acid (GABA)_A receptor complex. BDZs, which are widely used as anxiolytic and anticonvulsant drugs, are agonists at BZR. Drugs have been found (termed inverse agonists) that produce effects opposite to the BDZ agonists (anxiogenic, proconvulsive effects) by inducing a conformation of the BZR that depresses GABA-mediated Cl-channel gating. There is ample evidence that flumazenil is able to antagonize effects of both BZR agonists and inverse agonists.

Flumazenil has been used in behavioral pharmacology not only as a tool to study effects of exogenous ligands of BZR but also as a probe to detect effects of putative endogenous ligands for BZR (endozepines; Rothstein et al. 1992; Malagon et al. 1993) in anxiety (“the endozepine” hypothesis). If putative endogenous ligands for BZR would exert an agonist action, then flumazenil alone could produce an anxiogenic effect (an “agonist” variant of the endozepine hypothesis). On the contrary, flumazenil would be expected to show an anxiolytic activity if the endogenous ligands have an inverse agonist activity (an “inverse agonist” variant of the endozepine hypothesis).

Chemicals that are active at the BZR are naturally present in the central nervous system. Some of them, such as diazepam binding inhibitor (DBI) and its processing products, including the octadecaneuropeptide (ODN), are polypeptides with inverse agonist activity at BZR (Bormann 1991; Costa and Guidotti 1991). Endozepines with agonist activity at BZR were also found (Rothstein

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et al. 1992), but knowledge on agonist endozepines is limited.

Flumazenil has not evoked significant anxiety in healthy volunteers under quiescent conditions (Darragh et al. 1983; Higgitt et al. 1986; Wolf et al. 1990; Ghoneim et al. 1993). The putative endogenous ligands for BZR_s might be mobilized only under certain situations (such as threat). For example, indices of increased activity of DBI were found in the brain after psychological stress in mice (Katsura et al. 2002) and rats (Sudakov et al. 2001). Consequently, flumazenil might reveal its specific action at anxiogenic situations or in anxious subjects.

Equivocal effects of flumazenil on anxiety or anxiety-like behavior have been found in man and in animals. Flumazenil decreased social interactions in rats, interpreted as an anxiogenic-like effect (File et al. 1982, 1986; File and Pellow 1984) but it was without anxiogenic activity in the elevated plus maze in rats (Pellow and File 1986; Baldwin and File 1988) or in rat pup ultrasonic vocalizations (Olivier et al. 1998). Although this could be explained by differences in experimental models, there are many examples of contradictory results of flumazenil effects on anxiety obtained with the same method. For example, disparate effects of flumazenil were found in the elevated plus-maze test in mice: flumazenil increased the time spent on the closed arms of the maze (an anxiogenic profile; Lee and Rodgers 1991), produced no effect (Dalvi and Rodgers 1999) and induced strong anxiolytic effect (Belzung et al. 2000). The inconsistencies in flumazenil effects on anxiety represent a problem in the investigation of putative endogenous ligands of BZR_s. Attempts have been made to explain the disparate effects of flumazenil on anxiety.

First, the disparity could be due to differences in dosage. It is generally believed that flumazenil may produce at low doses mild inverse agonist-like effects while the agonist-like effects of flumazenil are expected as the dose is increased. This hypothesis (the "dose-related" hypothesis) is well founded by results of *in vitro* studies. Thus, flumazenil moderately inhibited GABA-induced current under voltage-clamp conditions at low concentrations (less than 1 μ M) and stimulated GABA current at higher concentrations (higher than 1 μ M, Sigel and Baur 1988). However, many reported studies of flumazenil effects on anxiety have not used a dose range of flumazenil broad enough to assess this relationship. Moreover, similar doses of flumazenil produced disparate effects in the same anxiety paradigm, for example 10 mg/kg of flumazenil was reported to be anxiogenic in the social interaction test in rats (File and Pellow 1984; File et al. 1986) as well as to be without effect (Quock et al. 1993).

Another hypothesis that attempts to explain the bidirectional effects of flumazenil on anxiety refers to the baseline level of anxiety. According to this ("anxiety-related") hypothesis, "the crucial factor is the anxiety level of the animal: when this is high flumazenil becomes anxiolytic; when this is low flumazenil is anxiogenic" (File and Hitchcott 1990). Regrettably, it is difficult to

compare anxiety levels of different experimental situations in different laboratories and few attempts have been made to test flumazenil effects under low and high anxiety levels in the same laboratory and measure of anxiety. In a mouse defense battery, flumazenil (20 mg/kg) increased avoidance distance to an approaching rat (a situation interpreted as a weakly threatening), indicating an inverse agonist-like action, while 5 mg/kg and 10 mg/kg of the drug decreased defensive biting under a forced contact to a rat (in a highly threatening situation) indicating an agonist activity (Griebel et al. 1995).

Disparate effects of flumazenil on anxiety could be also due to individual differences in tested subjects (the "individual differences related" hypothesis). However, the relationship between flumazenil effects and individual characteristics (e.g. behavioral, genetic, biochemical) have been studied rarely so far. Flumazenil produced anxiolytic effects in the elevated plus maze and the light/dark test in BALB/c mice but not in C57BL/6 mice (Belzung et al. 2000). Significant differences in brain levels of DBI and other peptides regulating anxiety were found in two inbred rat strains exhibiting different levels of anxiety (Sudakov et al. 2001).

Considering the complexity of factors that can influence flumazenil effects (and which hamper an assessment of the role of endozepines in anxiety), we thought it might be worth investigating again the behavioral effects of flumazenil under situation with strong anxiogenic and natural stimulus producing different levels of anxiety-like behavior in different types of subjects. Therefore, the aim of the present study was to ascertain effects of flumazenil in the social conflict test in mice, which provides complex measures for prediction of anxiolytic and anxiogenic activity of drugs in subjects with different levels of anxiety-like behavior. The social conflict test in mice (Kršiak 1975, 1979; Kršiak et al. 1984) is one of the ethologically oriented variants of animal models of anxiety based on social behavior. Analysis of defensive-escape, aggressive, social and locomotor behaviors occurring during social encounters between male mice enables assessment of drug effects on anxiety and aggression as well as a degree of motor impairment and sedation (Kršiak 1975, 1979; Miczek and Kršiak 1979; Kršiak et al. 1984). It was previously shown that BZR_s ligands show characteristic behavioral profiles in the social conflict test in mice: agonists reduced defensive-escape and aggressive behavior and increased (disinhibited) social investigation and/or locomotion while inverse agonists produced to some extent opposite effects (Kršiak 1975, 1979; Kršiak et al. 1984; Kršiak and Šulcová 1990; Šulcová et al. 1992). Moreover, the model allows the comparison of behavioral effects of drugs in different populations of mice (Kršiak 1976): predominantly timid (exhibiting defensive postures or escapes even though their partners are non-aggressive), predominantly aggressive (exhibiting attacks) or predominantly sociable (exhibiting social investigation but no defenses, escapes or attacks). These three types of mice might differ not only in the proportion of anxiety-like behavior, but also in the

brain chemistry. Given the close relationship of endozepines and BZR to GABA action, an additional objective of the present study was to examine concentrations of GABA in the brain of timid, aggressive and sociable mice by microdialysis *in vivo*.

There is only limited knowledge on the effects of flumazenil on defensive-escape agonistic behavior occurring during social conflict. Flumazenil (1.25–20 mg/kg) did not change defensive behavior significantly in a resident-intruder conflict in male mice, with the exception of a component “under food hopper” which was reduced after 1.25 mg/kg of the drug (Rodgers and Waters 1984). In other published studies, only data of a single dose of flumazenil on conspecific defense are available: flumazenil had either no effects on agonistic defensive behavior in mice (20 mg/kg, Kršiak et al. 1984; 5 mg/kg, Šulcová and Kršiak 1990), or increased it (10 mg/kg, Poshivalov 1987).

The present experiment examined effects of a wide dose range of flumazenil (5–80 mg/kg) on a broad spectrum of behavior occurring during social conflict in three categories of mice (aggressive, timid, and sociable subjects). In particular, we wanted to examine whether flumazenil produced anxiolytic-like or anxiogenic-like behavioral changes in this model and, if so, to assess the role of dosage, anxiety level, individual differences and putative endozepines in these behavioral changes.

Materials and methods

Subjects

Male albino random-bred mice, derived from ICR strain (Velaz, Prague, Czech Republic) weighing 18–20 g at the beginning of the experimental housing were used. They were housed singly in self-cleaning cages or in groups of ten. The cages used for the individual housing were made of solid metal walls 13 cm high with wire-mesh floors (8×17 cm), which were placed 3 cm above trays with wood shavings. This wire-mesh floor ensured that the isolates were not handled throughout the period of single housing. The mice kept in groups were housed in large standard plastic cages (26×42×15 cm) with floors covered with wood shavings. All mice were housed under room lighting (with lights on from 0600 hours to 1800 hours) and under temperature ranging from 22°C to 24°C. Food and water were available *ad libitum*.

The mice were observed in transparent cages (20×30×20 cm) with wood shavings on the floor and tops covered with transparent covers with apertures for air. The observations were performed under moderate room lighting from 0800 hours to 1300 hours.

Experiments were approved by the Expert Committee for Protection of Experimental Animals of the 3rd Faculty of Medicine, Charles University in Prague and were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb).

Experimental procedures

Social conflict test

Social interaction tests started after 3 weeks of isolation and always involved one singly housed mouse paired with the same group-housed mouse. The isolates were allowed 15 min adaptation in the observational cages before the group-housed partners were intro-

duced; the interaction ended after 4 min. This procedure, which suppresses aggression in group-housed mice and reduces their social behavior, facilitates active social behavior in isolates. The observation cages were cleaned and their floors were covered with new wood shavings after each interaction.

All subjects underwent four social interaction tests at 1-week intervals. The isolates were given a particular dose of flumazenil or vehicle in a randomized order, according to a Latin square design (each mouse served as its own control), while the group-housed partners remained untreated. The group-housed mice served only to stimulate social behavior in the isolates. In the case that a group-housed ‘stimulus’ mouse attacked the isolated mouse, the pair was excluded from the experiment.

The behavior of animals during the interactions was recorded on videotape. The tapes were later analyzed by an observer with no knowledge of the drug treatment. This was done with a key-board that was connected to a standard PC and a software for behavioral analysis (Donát 1991).

Measures

The frequency, total duration and latency of a number of aggressive, defensive-escape (timid), social and locomotor activities derived from the ethogram of mice (Grant and Mackintosh 1963) and described in detail previously (Kršiak 1975, 1979; Votava et al. 2001) were recorded.

Sociable activities, acts (social investigation)

Sociable activities included the social sniff—sniffing the partner’s head, body, genitals or tail; climb—the mouse places its forepaws on the partner’s back, mostly in the shoulder region, and usually sniffs this area at the same time; and follow—following the partner by quiet walking.

Aggressive activities (acts)

Aggressive activities included attack—a fierce lunging at the partner often associated with biting; threat—a sideways or an upright stance with head and forebody movements toward the partner, and trying to bite the partner (offensive sideways or upright posture); and tail rattle—rapid vibrations of the tail.

Timid activities (acts)

Timid activities included defense—the mouse responds to the partner’s social behavior by raising forepaws, hunching the back (defensive upright posture) or by some rotation of the body bringing the legs closest to the other animal off the ground (defensive sideways posture); escape—a rapid running or jumping away from the partner; and alert posture—a sudden interruption of all movements with eyes and ears being directed toward the partner.

Locomotor activities (acts)

Locomotor activities included walk—any walking across the cage that is not apparently related to the partner; and rear—the mouse stands only on his hind legs and usually sniffs air or walls at the same time.

Duration was not measured for escapes and attacks because of momentary character of these acts (i.e. measurement of duration was not considered accurate enough and meaningful in these acts).

The interobserver reliability of the recorded items was satisfactory as determined by several observers independently scoring a videotaped record of behavior of 70 mice in interactions lasting 4 min each. The correlation ranged from $r=0.83$ to $r=0.97$.

Measurement of GABA

Schedule of experiment

After 3 weeks of isolation, a social interaction was performed with a separate group of undrugged isolates under conditions described above. According to behavior exhibited during the interaction, the isolates were classified into three groups: aggressive, sociable and timid mice (see below). One week later, the microdialysis assay was performed in the isolates. The medial prefrontal cortex of mice was dialyzed and basal concentrations of GABA in the dialysates were subsequently evaluated using the CE LIF (see below).

Probe preparation and surgery

Concentric dialysis probes were prepared by a modification of the method described by Fišerová et al. (1999). Each probe was assembled from dialysis fiber of AN 69 (sodium sulfate copolymer) (20,000 Da, 310 μm OD, 220 μm ID; Hospal, Dasco, Bologna, Italy), silica-fused capillary tubing (ID 75 μm ; OD 150 μm), 30-gauge stainless-steel cannula, polyethylene tubing (ID 0.58 mm; OD 0.96 mm) using Super-Epoxy. The proper dialysis-open area on the dialysis fiber was 1 mm (from the tip). Under Equithesin anesthesia (1% pentobarbital and 4% chloral hydrate), mice were implanted with the vertical dialysis probes, using a stereotaxic apparatus. After taking the co-ordinates with the dialysis probe mounted on the stereotaxic holder (medial prefrontal cortex; mPFC: A: +2.5 mm and L: ± 1.3 mm from bregma and V: 5.0 mm from occipital bone) (Franklin and Paxinos 1996) the fiber was slowly lowered into the brain and secured to the skull with dental cement. After completion of the microdialysis experiments, the placement of the dialysis probe was verified histologically. Animals with the probe outside the mPFC region were discarded.

Microdialysis and biochemistry

At least 24 h after implantation, the dialysis probe was perfused at a constant rate of 2 $\mu\text{l}/\text{min}$ with Ringer's solution. The dialysate was discarded during the first 30 min of dialysis. Subsequently, 40- μl samples were collected at 20-min intervals in small ice-cooled polyethylene test tubes. The baseline samples were collected during the 80-min equilibration period to allow the neurotransmitter to reach the steady level. Immediately following collection, the samples were frozen on dry ice and lyophilized. GABA contents were quantified by a specific and selective analytical method using capillary electrophoresis with laser-induced fluorescence detector (CE LIF); the concentration of GABA is given in pmols/20 min. In vitro recovery of the neurotransmitter always through three dialysis probes (7.5 mm) was determined; the average recovery of GABA was 50.3 \pm 1.7%.

Drugs and reagents

Flumazenil (Ro 15-1788, Hoffman-La Roche) was dissolved in saline with two drops of Tween 80 and administered intraperitoneally in a volume 0.1 ml/10 g of body weight 15 min before interaction. Immediately after the administration, isolated mice were placed into the observational cage for a 15-min acclimation period. A wide dose range (5, 20, and 80 mg/kg) was tested; a relatively high dose (80 mg/kg) was used in order to see whether flumazenil still produces only partial effects even at this high dose. A pilot study showed that 3.5 mg/kg flumazenil was ineffective in the present experimental setting.

All reagents were of analytical grade. The Ringer's solution (147 mM NaCl, 2.2 mM CaCl₂ and 4.0 mM KCl adjusted to pH 7.4 with NaOH 0.1 N) was used for dialysis. Gamma-amino-*N*-butyric acid (GABA; 4-amino-*n*-butyric acid) was purchased from Sigma-Aldrich Co.

Data analysis

The isolates were first classified into three groups according to their behavior in the control interaction: aggressive mice (exhibiting attacks), timid mice (exhibiting escapes or defensive postures but no attacks), and sociable mice (exhibiting no attacks, defenses or escapes). The procedure used to classify the mice was described previously (Kršiak 1975). The individual type of behavior (aggressive, timid, sociable) has been significantly stable in repeated interactions as shown previously (Kršiak 1975; Donát 1986; Kršiak and Šulcová 1990). The incidence of some activities may change over repeated interactions. For this reason, the order of treatments was randomized according to a Latin square design. Three isolates were excluded from the experiment because their partners attacked them.

Behavioral elements (acts), their frequency, duration, and latency were summed in four behavioral categories (sociable, aggressive, locomotor, and timid) for the statistical analyses. The behavioral categories were evaluated using a one-way, repeated-measures analysis of variance (ANOVA; with the factor treatment) separately in aggressive, timid, and sociable mice. Subsequent analysis was performed using Bonferroni *t*-test to reveal significant differences between the control and flumazenil treatment. If data were of a non-parametric nature, they were analyzed using Friedman repeated-measures ANOVA on Ranks and post-hoc comparisons between the control and flumazenil treatment were performed using Dunnett's test.

The concentrations of GABA in the dialysates from brains of the three categories of mice were statistically analyzed using a one-way ANOVA followed by Bonferroni *t*-test for multiple comparisons.

Results

Social conflict test

Behavior in the control interaction

Twenty-one singly housed males (53.9%) did not attack their partners but showed a greater number of defensive postures, escapes, and alert postures (timid acts, Fig. 1), even though their group-housed partners were completely non-aggressive. These isolates are therefore called the "timid" isolates (Kršiak 1975). The second category of isolates ($n=11$, 28.2%) attacked their partners in the control interaction. The aggressive isolates showed also a number of threats, tail rattles (aggressive acts) and locomotor acts (Fig. 2). The rest of the isolates ($n=7$, 17.9%), which did not show attacks or defenses or escapes, exhibited a greater amount of social investigation (sociable acts, Fig. 3). They are called the "sociable" isolates.

The activity of group-housed partners (the "stimulus" mice) was largely composed of locomotion (walking across cage and rearing), while their active social behavior was limited to a smaller amount of approaching and sniffing the isolates. Defensive postures and escapes occurred in the group-housed mice only when interacting with aggressive isolates as passive responses to aggressive behavior of their partners. If a "stimulus" male did attack the isolate, which occurred very rarely, the whole pair was excluded from the study.

Timid mice

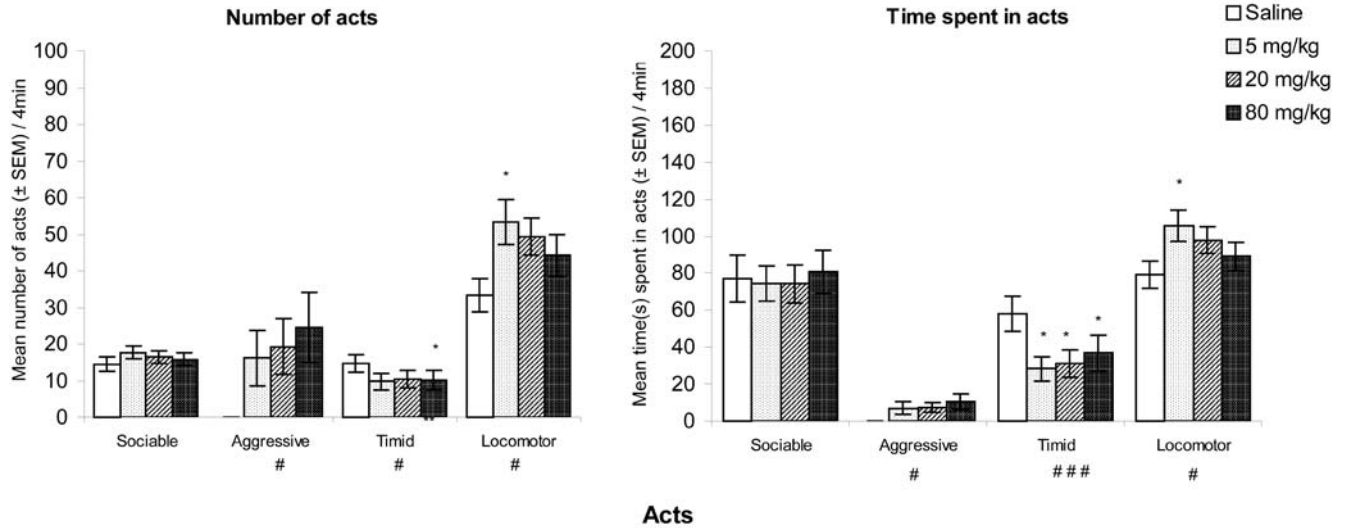


Fig. 1 Effects of flumazenil on behavior (frequencies and duration of acts) of timid mice ($n=21$) in the social conflict test. One-way, repeated-measures ANOVA ($\#P<0.05$, $\##P<0.01$, $\###P<0.001$) with

subsequent comparisons with the saline-treated group ($*P<0.05$, $**P<0.01$, $***P<0.001$)

Table 1 Effects of flumazenil on latencies to acts in the social conflict test. Mean latencies in seconds (\pm SEM) are shown. One-way, repeated-measures ANOVA ($\#P<0.05$, $\##P<0.01$, $\###P<0.001$) with subsequent comparisons with the saline-treated group ($*P<0.05$, $**P<0.01$, $***P<0.001$)

Mice	Acts	Saline	Flumazenil (mg/kg)		
			5	20	80
Aggressive $n=11$	Sociable	109.5 \pm 18.1	82.2 \pm 11.3	55.6 \pm 12.9	92.3 \pm 11.0
	Aggressive $\###$	12.8 \pm 6.37	170.8 \pm 28.3 $***$	139.2 \pm 31.0 $**$	109.0 \pm 30.7
	Timid	188.3 \pm 7.0	177.0 \pm 18.0	196.2 \pm 15.2	181.6 \pm 11.3
	Locomotor	40.5 \pm 7.9	77.1 \pm 21.9	60.5 \pm 13.7	46.5 \pm 13.1
Timid $n=21$	Sociable	118.9 \pm 11.9	106 \pm 11.5	112.3 \pm 12.0	111.3 \pm 11.0
	Aggressive $\#$	239.9 \pm 0.1	197.4 \pm 17.8	164.5 \pm 21.4	168.6 \pm 22.7
	Timid	88.6 \pm 11.0	133.1 \pm 13.7	119.9 \pm 16.5	128.0 \pm 18.4
	Locomotor	37.8 \pm 7.8	41.7 \pm 7.2	33.9 \pm 5.8	44.5 \pm 6.7
Sociable $n=7$	Sociable	62.6 \pm 13.4	57.4 \pm 12.7	68.4 \pm 11.9	60.4 \pm 11.8
	Aggressive $\#$	231.0 \pm 8.3	157.8 \pm 37.7	240.0 \pm 0.0	240.0 \pm 0.0
	Timid $\##$	206.7 \pm 12.0	183.8 \pm 9.5	143.5 \pm 17.6 $**$	194.8 \pm 14.8
	Locomotor $\#$	59.0 \pm 9.2	24.6 \pm 5.2	46.6 \pm 14.1	90.6 \pm 18.8

Effects of flumazenil

Timid mice

The effect of flumazenil on social behavior of timid mice is summarized in Fig. 1 and Table 1.

Timid activities. A Friedman repeated-measures ANOVA on ranks showed a significant effect of treatment in the number of timid acts (defenses, escapes, alert postures) ($\chi^2_{(3)}=9.396$, $P=0.024$) and in the time spent in timid acts ($\chi^2_{(3)}=16.892$, $P<0.001$, Fig. 1). Subsequent Dunnett's test showed a significant reduction of the number of timid activities after 80 mg/kg flumazenil ($P<0.05$, Fig. 1). Flumazenil also shortened the mean duration of timid activities at all doses tested (5, 20 and 80 mg/kg, $P<0.05$, Fig. 1).

Aggressive activities. A Friedman repeated-measures ANOVA on ranks showed a significant effect of treatment in the number of aggressive acts (attacks, threats, tail rattles) ($\chi^2_{(3)}=10.546$, $P=0.014$), in the time spent in aggressive acts ($\chi^2_{(3)}=10.299$, $P=0.016$, Fig. 1) and in latencies to aggressive acts ($\chi^2_{(3)}=10.748$, $P=0.013$, Table 1). However post-hoc comparisons with the control treatment yielded no significant values in any of these parameters in any dose of flumazenil.

Locomotor activities. A one-way, repeated-measures ANOVA exhibited a significant effect of treatment in the number of locomotor activities (walk, rears) ($F_{3,60}=3.852$, $P=0.014$, Fig. 1) as well as in the time spent in locomotor acts ($F_{3,60}=3.334$, $P=0.025$; Fig. 1).

Subsequent Bonferroni t -tests showed a significant increase of the number of locomotor activities after administration of 5 mg/kg flumazenil ($t=3.207$, $P=0.013$).

Aggressive mice

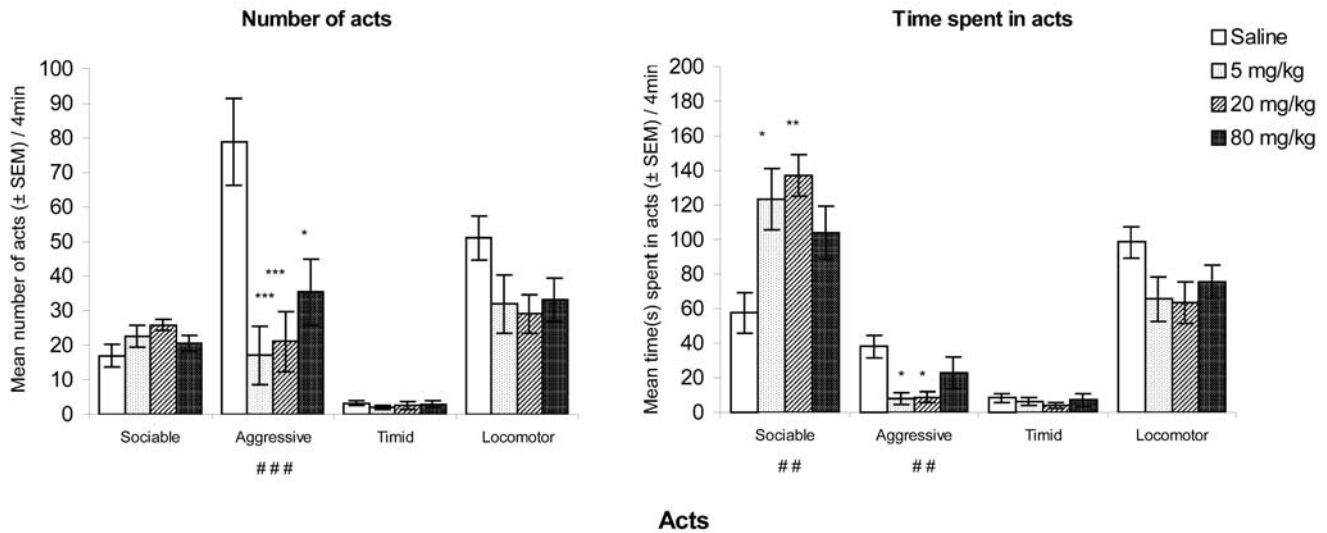


Fig. 2 Effects of flumazenil on behavior (frequencies and duration of acts) of aggressive mice ($n=11$) in the social conflict test. One-way, repeated-measures ANOVA ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$)

with subsequent comparisons with the saline-treated group ($*P<0.05$, $**P<0.01$, $***P<0.001$)

The administration of 5 mg/kg flumazenil also prolonged the duration of locomotor activities ($t=2.999$, $P=0.024$; Fig. 1).

Sociable activities. No significant changes were found in any of the measures of sociable activities.

Aggressive mice

The effect of flumazenil on social behavior of aggressive mice is summarized in Fig. 2 and Table 1.

Aggressive activities. A one-way, repeated-measures ANOVA showed a significant effect of treatment in the number of aggressive acts (attacks, threats, tail rattles) ($F_{3,30}=9.705$, $P<0.001$; Fig. 2) and in latencies to aggressive acts ($F_{3,30}=7.406$, $P<0.001$; Table 1). Subsequent Bonferroni t -tests showed a significant reduction of the number of aggressive activities after administration of 5, 20 and 80 mg/kg of flumazenil ($t=4.812$, $P<0.001$; $t=4.5$, $P<0.001$, and $t=3.38$, $P=0.012$, respectively; Fig. 2). The administration of 5 mg/kg and 20 mg/kg flumazenil prolonged the latency to aggressive activities ($t=4.453$, $P<0.001$ and $t=3.564$, $P=0.007$, respectively; Table 1).

A Friedman repeated-measures ANOVA on ranks revealed a significant effect of treatment in the time spent in aggressive acts ($\chi^2_{(3)}=11.272$, $P=0.01$; Fig. 2). Subsequent Dunnett's tests confirmed that 5 mg/kg and 20 mg/kg flumazenil significantly shortened the total duration of aggressive activities ($P<0.05$, Fig. 2).

Sociable activities. A one-way, repeated-measures ANOVA showed a significant effect of treatment in the time spent in sociable acts (social sniffs, climbs, follows) ($F_{3,30}=5.028$, $P=0.006$; Fig. 2). Subsequent Bonferroni t -tests revealed a significant prolongation of the mean time spent in sociable activities after administration of 5 mg/kg and 20 mg/kg flumazenil ($t=3.004$, $P=0.032$ and $t=3.632$, $P=0.006$, respectively; Fig. 2).

Locomotor and timid activities. No significant changes were found in any of the measures of timid and locomotor activities.

Sociable mice

The effect of flumazenil on social behavior of sociable mice is summarized in Fig. 3 and Table 1.

Sociable activities. Although one-way, repeated-measures ANOVA showed a significant effect of treatment in the time spent in sociable acts ($F_{3,18}=3.385$, $P=0.041$; Fig. 3), subsequent comparisons of effects of individual flumazenil doses to the control treatment on duration of sociable acts were not significant. Similarly, no significant effects of flumazenil were found in frequencies and latencies to sociable acts.

Timid activities. A one-way, repeated-measures ANOVA showed a significant effect of treatment on latencies to timid acts ($F_{3,18}=6.209$, $P=0.004$; Table 1). Subsequent Bonferroni t -tests showed that the shortening of latency to timid acts after 20 mg/kg flumazenil was significant ($t=4.057$, $P=0.004$; Table 1). Furthermore, the Friedman

Sociable mice

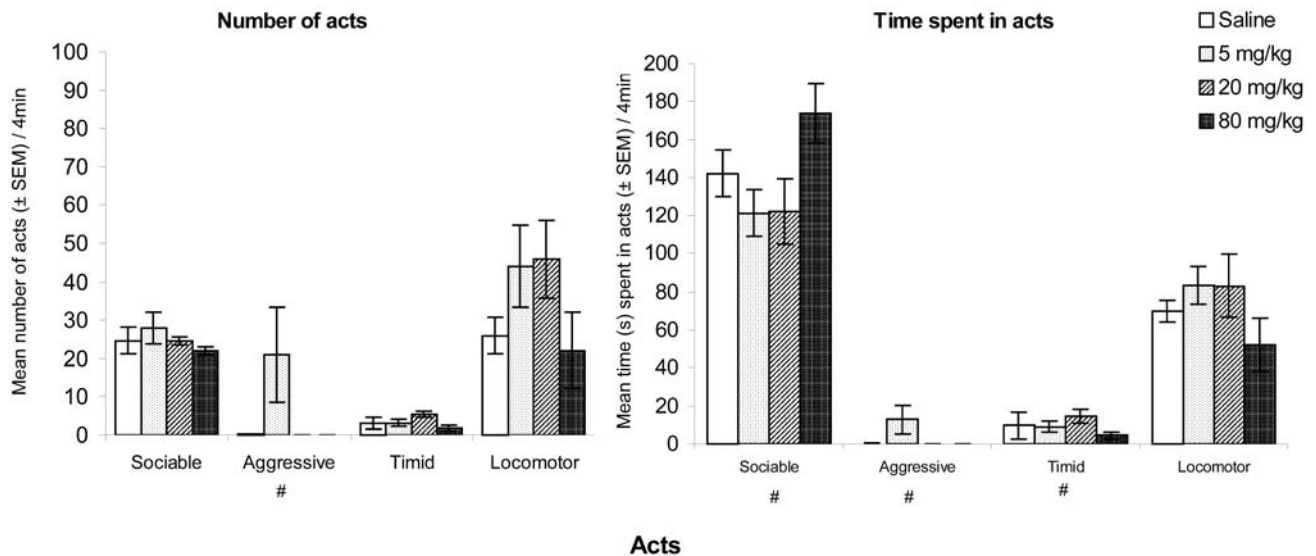


Fig. 3 Effects of flumazenil on behavior (frequencies and duration of acts) of sociable mice ($n=7$) in the social conflict test. One-way, repeated-measures ANOVA ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$) with subsequent comparisons with the saline-treated group ($*P<0.05$, $**P<0.01$, $***P<0.001$)

Table 2 γ -Aminobutyric acid (GABA) concentrations (mean \pm SEM) in dialysates from the medial prefrontal cortex in three categories of mice. One-way ANOVA ($P<0.001$) and P for subsequent multiple comparisons with Bonferroni test

	n	GABA pmol/40 μ l	P value	Comparison
Timid mice	6	64.10 \pm 11.59	1.000	Timid vs aggressive mice
Aggressive mice	10	64.05 \pm 8.09	1.000	Aggressive vs timid mice
Sociable mice	10	189.24 \pm 22.49	<0.001	Sociable. vs timid mice/ sociable vs aggressive mice

repeated-measures ANOVA on ranks showed a significant effect of treatment in the time spent in timid acts ($\chi^2_{(3)}=7.8$, $P=0.05$; Fig. 3). However, no significant results were found in statistical evaluation of frequencies of timid acts.

Aggressive activities. Although the Friedman repeated-measures ANOVA on ranks showed a significant effect of treatment in the number of aggressive acts (attacks, threats, tail rattles) ($\chi^2_{(3)}=8.143$, $P=0.043$; Fig. 3), the time spent in aggressive acts ($\chi^2_{(3)}=8.143$, $P=0.043$; Fig. 3) as well as in latencies to aggressive acts ($\chi^2_{(3)}=8.143$, $P=0.043$; Table 1), subsequent comparisons of effects of individual flumazenil doses to the control treatment revealed no significant changes.

Locomotor activities. Statistical analysis indicated a significant effect of treatment only in latencies to locomotor acts ($F_{3,18}=5.149$, $P=0.01$; Table 1).

GABA concentrations

There was a significant difference in the mean values of GABA concentrations in dialysates from the medial

prefrontal cortex among timid, aggressive and sociable mice ($F_{2,23}=20.008$, $P<0.001$, one-way ANOVA, Table 2). Levels of GABA were markedly and significantly higher in the medial prefrontal cortex of sociable mice than those in timid and aggressive mice ($t=5.703$ and 4.937 , respectively, $P<0.001$; Bonferroni t -test). No differences were found between GABA levels in dialysates from brains of timid and aggressive mice (Table 2).

Discussion

Flumazenil produced an anxiolytic-like profile of behavioural effects in timid and aggressive mice: the drug reduced timid (defensive-escape) and increased locomotor activities in timid mice and reduced aggressive and increased sociable activities in aggressive mice. A slight anxiogenic-like effect was found after flumazenil in sociable mice.

Flumazenil produced the anxiolytic-like behavioural effects within the whole range of doses tested (5–80 mg/kg). Although we cannot exclude that lower doses of flumazenil might be anxiogenic in the present experimental model, this seems unlikely because markedly lower doses of flumazenil (0.0001–1 mg/kg) still pro-

duced anxiolytic effects in the elevated plus maze test in mice (Belzung et al. 2000). There appeared to be some tendency to increased timidity in sociable mice, but this was significant only at the medium dose (20 mg/kg) and in one parameter (the latency to timid acts). Thus, we were unable to find evidence for the “dose-related” hypothesis of bidirectional effects of flumazenil on anxiety in terms of anxiogenic activity at low doses and anxiolytic action at high doses in the present study.

Timidity measures were low in aggressive and sociable mice and high in timid mice. According to the “anxiety-related” hypothesis, anxiolytic effect of flumazenil would be expected in timid mice and the anxiogenic one in aggressive or sociable mice. While the anxiolytic-like effect in terms of reduced timidity and disinhibited locomotion and/or sociability of flumazenil was well represented in timid mice, the anxiogenic effect was slight in sociable mice and missing in aggressive mice (flumazenil actually produced anxiolytic-like changes in aggressive mice—see below).

Although anxiety level at its extremes may influence flumazenil effects on anxiety, individual differences seem to be as important for the final outcome of flumazenil action on anxiety. All mice were randomly selected and subjected to the same social isolation and treatment, but only some responded to a strange partner by timid or aggressive behavior. Those who did not (sociable mice) were responding differently to flumazenil. There seem to be differences in brain chemistry between sociable and aggressive or timid mice: sociable mice had markedly higher levels of GABA in the forebrain than aggressive and timid mice as determined by microdialysis *in vivo*. Flumazenil may show discrepant effects on anxiety in different subjects even when they show similar behavior. Flumazenil (2 mg *i.v.*) was reported to produce panic attacks in 8 of 10 panic disorder patients in one study (Nutt et al. 1990) but in none of eight panic disorder patients in another study (Strohle et al. 1999). Thus, even under comparable conditions as for the dosage or psychic disorder there seem to be subjects differently responding to flumazenil effects on anxiety. Additional research is necessary to find whether flumazenil can serve as a marker to detect such individuals and what biochemical or genetic mechanisms might underlie these individual differences.

Incidence of representatives of the three categories of mice classified by the present procedure (and possibly subjects with different sensitivity to flumazenil effects) may vary to some extent in separate deliveries of random bred mice. The prevalence of timid mice, aggressive mice, and sociable mice has been 36–53%, 28–47% and 2–18%, respectively, during the 30-years period that we have used the present procedure unchanged (Kršiak 1975, 1976; Kršiak et al. 1984; Kršiak and Šulcová 1990; Podhorná and Kršiak 2000). Regrettably, intensity of the active defensive-escape behavior (timidity) appears to have been declining during this period (probably due to domestication). Wild mice exhibit much stronger defen-

sive-escape activity (Holmes et al. 2000; Blanchard et al. 2001; Hendrie et al. 2001).

In the present study, flumazenil reduced timidity and aggression and increased locomotion and social investigation even at the lowest dose tested (5 mg/kg), and these effects were not increased further by higher doses of the drug (20 mg/kg or 80 mg/kg). A question arises whether the present results in timid and aggressive mice reflect an intrinsic partial agonist activity of flumazenil *per se* or rather its antagonism of putative endogenous ligands of BZR_s. In the latter case, the endogenous ligands would have a partial inverse agonist activity. Although flumazenil was shown to enhance the GABA_A-receptor mediated chloride currents at high concentrations *in vitro* (Weiss et al. 2002), results of *in vivo* simultaneous determination of fractional BZR_s occupancy estimated by positron emission tomography and the pharmacological efficacy measured as an ability to influence convulsant activity of pentetrazol on electroencephalogram (EEG) in the brain of non-human primates (baboons) have not brought evidence of positive intrinsic efficacy in flumazenil (Brouillet et al. 1991). Also in another *in vivo* study (in rats), flumazenil did not produce changes of EEG in concentration range of receptor saturation (Mandema et al. 1992). If flumazenil would exert partial agonistic activity *per se* under conditions of *in vivo* experiments, then it would not antagonize effects of other partial agonists such as bretazenil. However, flumazenil has been reported to antagonize antisuppressant effects of bretazenil on punished responding in monkeys (Paronis and Bergman 1999).

The present results in timid and aggressive mice are not in themselves evidence of antagonism of endogenous anxiogenic-like BZR ligands by flumazenil, but they can be accounted for in this way. Partial inverse BZR agonists (beta-CCE and FG 7142) increased timid activities (defenses, alert postures) in the present model (Šulcová et al. 1992), and flumazenil antagonized these effects (Šulcová and Kršiak 1990). A shorter fragment of DBI, such as the ODN (Ferrero et al. 1986), with inverse agonist activity at BZR_s (Costa and Guidotti 1991; Rouet-Smith et al. 1992), produced anxiogenic effects in mice and these effects were antagonized by flumazenil (Mateos-Verchere et al. 1998).

While the anxiolytic-like effects of flumazenil in timid and aggressive mice are in agreement with the “inverse agonist” variant of the “endoneurypine hypothesis”, the slight anxiogenic-like effect of flumazenil observed in sociable mice might be interpreted as reflecting an antagonism of endoneurypines with agonist activity. It is conceivable, that both types of endoneurypines might be activated in the anxiogenic situation (the “dual agonist” variant of the “endoneurypine hypothesis”). The final outcome (in behavior, emotion and flumazenil effects) would then depend on relative predominance of one of the opposite endoneurypines. At present, it is unknown whether the presumed higher activity of agonist endoneurypines in sociable mice and the observed higher levels of GABA

in their forebrains are related to the low timidity shown by these animals during social encounters.

Thus, the current results might be reflecting antagonism of endogenous ligands for BZR. The putative endogenous BZR ligands, if any, seem to play only a modulatory or partial role in the present model of anxiety, however, because flumazenil was not able to inhibit agonistic defensive-escape behavior fully or to produce marked anxiogenic-like changes. The higher doses of flumazenil tested (20 mg/kg and 80 mg/kg) greatly exceeded those reported to compete effectively with other ligands for the BDZ recognition site (Martin et al. 1993). BDZ full agonists were able to inhibit fully agonistic defensive-escape behavior in the present model only at very high doses producing gross motor impairment and overt sedation (Kršiak et al. 1984; Podhorná and Kršiak 2000). In fact none of more than 50 drugs from various pharmacological classes tested in the present model was able to inhibit fully defensive-escape agonistic behavior at non-toxic doses (Kršiak et al. 1984). A drug of that kind remains to be discovered.

Flumazenil reduced aggression in aggressive mice at a wide dose range in the present study. In contrast, flumazenil was reported to exert little or no effects on agonistic aggressive behaviors in confrontations with conspecifics in mice, rats, and squirrel monkeys when administered alone (Rodgers and Waters 1984; Skolnick et al. 1985; Mos and Olivier 1986; Weerts et al. 1992, 1993b), although in one study it reduced resident-intruder confrontations in rats (Weerts et al. 1993a). Inverse agonists beta-CCE and FG 7142 also reduced aggression in the present test situation (Šulcová et al. 1992). This may appear in disagreement with the hypothesis on inverse agonist character of the putative endogenous ligands presumably antagonized by flumazenil suggested above in the context of timid behavior. However, the reduction of aggression after beta-CCE and FG 7142 was associated with an increase of timid behavior (Šulcová et al. 1992) while the reduction of aggression after flumazenil was associated with an increase in sociability (social investigation) in the present study. It is conceivable that in the present experimental situation (meeting a strange male in a strange environment), fear might both facilitate and inhibit aggression. Thus, the aggressive mice could be less aggressive after flumazenil because they might be less fearful after inhibition of effects of the presumed endogenous inverse BZR agonists by flumazenil. They could be less aggressive after exogenous inverse agonists such as beta-CCE and FG 7142 because they might be more fearful after an increase of effects of the presumed endogenous inverse BZR agonists by the exogenous inverse agonists. Flumazenil antagonized both "anxiogenic" and anti-aggressive effects of FG 7142 in the present experimental model (Šulcová and Kršiak 1990).

In conclusion, flumazenil in a wide dose range produced anxiolytic-like behavioural profile in timid and aggressive mice and a slight anxiogenic-like effect in sociable mice in the social conflict. The present results suggest that individual differences rather than dosage

determine flumazenil effects on anxiety. Although anxiety appears to be a prerequisite for manifestation of flumazenil effects on anxiety, individual differences might be as important for the final outcome of flumazenil effects on anxiety. The present data might be reflecting antagonism of endogenous BZR ligands released in response to the threatening nature of the social encounter. However, the putative endogenous ligands seem to exert only a modest modulatory influence on behaviors displayed during these agonistic interactions.

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