JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1997, 48, 2, 253—261

P. POKK, A. ZHARKOVSKY

THE EFFECTS OF FLUMAZENIL, RO 15-4513 AND β-CCM ON THE BEHAVIOUR OF CONTROL AND STRESSED MICE IN THE PLUS-MAZE TEST.

Department of Pharmacology, University of Tartu, Tartu, Estonia

The effects of the benzodiazepine receptor antagonist flumazenil (Ro 15-1799), the benzodiazepine receptor partial inverse agonist Ro 15-4513 and the benzodiazepine receptor inverse agonist β-CCM on the behaviour of control and small platform stressed mice studied. Small platform stress was induced by placing the animals on small platforms (d = 3.5 cm) surrounded by water for 24 hours. This technique involves several factors of stress such as rapid eye movement sleep deprivation, isolation, immobilization, falling into the water and soaking.

In the plus-maze test small platform stress induced changes indicating anxiolytic action — an increase of the percentage of entries made onto and the percentage of time spent on the open arms. In control mice flumazenil (2.0 and 10.0 mg/kg), Ro 15-4513 (0.5; 1.0; 2.5; 5.0 and 10.0 mg/kg), and β-CCM (1.0 and 2.0 mg/kg) exerted dose-dependent anxiogenic effect. The small platform stress induced an enhancement of the anxiogenic effect of flumazenil, but not that of Ro 15-4513 and β-CCM. The selective enhancement of flumazenil's action may be explained with the mode of action of flumazenil. It is proposed that small platform stress causes changes in the concentration of the endogenous benzodiazepine receptor ligand with stress protective activity and flumazenil acts by blocking the effects of this endogenous ligand.

Key words: *stress, sleep deprivation, plus-maze, flumazenil, Ro 15-4513, β-CCM*

INTRODUCTION

The gamma-aminobutyric acid $(GABA)_A$ receptor-chloride ionophore complex undergoes rapid and persistent changes in the brain of stressed animals (1—3).

Consequently, behavioural effects of drugs acting at the $GABA_A$ receptor-chloride ionophore complex are different in stressed animals as compared to unstressed animals (4). As a rule, benzodiazepine receptor agonists inhibit the behavioural consequences of the stress exposure, and the benzodiazepine receptor inverse agonists enhance them (5, 6).

Various acute chronic models of stress exert anxiogenic effect in laboratory animals. In rats and mice acute stress (handling, exposure to noise, footshock, etc.) reduces locomotor activity (7, 8) and induces anxiogenic effect in the plus-maze (9, 10) and hole-board tests (8, 11).

Chronic exposure of laboratory animals to mild unpredictable stressors during 11—38 days produces a generalized decrease in sensitivity to reward (anhedonia) as assessed by reduced consumption of sucrose solution (12, 13), ventral tegmental self-stimulation behaviour (14), and inhibition of food-induced place preference in rats (15).

It has been repeatedly demonstrated that animals exposed to the small platform surrounded by water for 24 hours or longer, undergo heavy stress. According to the original idea, animals exposed to the small platform stress will be selectively deprived of rapid eye movement sleep (16, 17). However, as this technique involves other factors of stress such as isolation, immobilization, falling into the water, this technique must be considered a model of stress where rapid eye movement sleep deprivation is one factor only (18, 19).

The peculiarity of small platform technique is that after stress exposure animals display increased locomotions and preference for novelty (18—20). The results of our previous experiments have also shown that in contrast to other stresses, mice exposed to small platform stress also exhibit anxiolytic-like behaviour in the plus-maze test (21, 22). Furthermore, the exposure to small platform stress induces changes in the functional activity of $GABA_A$ receptor-chloride ionophore complex: an increase in the number of cortical benzodiazepine receptors and reduction in the GABA-stimulated $^{36}Cl^-$ uptake by brain microsacs (23). The behavioural studies have demonstrated a decreased sensitivity to the anxiolytic effect and increased sensitivity to the sedative effect of diazepam and ethanol in animals exposed to small platform stress (21, 22). It is hypothesized that small platform stress-induced changes may be responsible for the modification of the effects of drugs acting at the $GABA_A$ receptor-chloride ionophore complex.

The aim of the present study was to investigate whether the behavioural effects of the benzodiazepine receptor antagonist flumazenil (Ro 15-1788), the partial inverse agonist Ro 15-4513 and the full inverse agonist β-CCM are changed after the small platform stress exposure.

MATERIALS AND METHODS

Animals

Naive male albino mice (NMRI strain, Grindex Breeding Center, Riga, Latvia) aged 5—6 months and weighing 25—40 g were used throughout the study. Mice were maintained at 20±2°C; water and standard laboratory food were available *ad libitum*. Mice were housed 20 per plastic cage and exposed to a 12/12 h light-dark cycle. Lights were on from 7.00 a.m. to 7.00 p.m.

Drugs

The benzodiazepine receptor antagonist flumazenil, the benzodiazepine receptor partial inverse agonist ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a] [1,4] benzodiazepine-3-carboxylate (Ro 15-4513) and the benzodiazepine receptor inverse agonist methyl-β-carboline-3-carboxylate (β-CCM) were kindly donated by Hoffman-La Roche, Basel, Switzerland. Flumazenil and Ro 15-4513 were suspended in saline with a drop of Tween-80, β-CCM was dissolved in 0.1 N HCl and then diluted with saline to volume 0.1 ml per 10 grams of body weight. All drugs were injected intraperitoneally. Flumazenil was injected 30 minutes, β-CCM 20 minutes and Ro 15-4513 — 5 minutes before behavioural testing. Control animals were given vehicle (saline with a drop of Tween-80 or diluted 0.1 N HCl) 5, 20 or 20 minutes before behavioural testing.

The small platform technique

Mice were exposed to the small platform stress for 24 hours according to the method described previously (24, 25) with some modifications. Mice were placed individually for 24 h on the small platform (3 cm high, 3.5 cm diameter) which was fixed at the center of a plastic chamber (20 cm diameter, 40 cm high) and surrounded by water (1 cm deep) at 22°C. Injections of drugs were made at the end of stress exposure. When drug or vehicle injections were carried out mice were returned to the platforms until behavioural testing. Animals were subjected to small platform stress starting at 12.00 a.m. for 24 h. Behavioural experiments were carried out immediately after the end of stress exposure.

To eliminate the effect of stress due to handling each animal was handled twice a day for 7 days before the start of experiments.

The plus-maze test

The plus-maze test was carried out according to File *et al.* (26). The plus-maze consisted of two open (8 × 17 cm) and two closed arms (8 × 17 × 30 cm), which were connected by a central platform (8 × 8 cm). Mice were placed on the central platform facing an open arm. During 5 minutes the number of entries made onto the open and into the closed arms and the time spent on the open arms were measured. On the basis of these data the percentage of entries made onto the open arms and the percentage of time spent on the open arms were calculated.

Statistics

Data were analyzed using two-way analysis of variance (ANOVA), where small platform stress was taken as one factor and effect of drugs as another. Post-hoc statistical analysis was made by the Bonferroni test.

RESULTS

The effect of flumazenil, Ro 15-4513 and β-CCM on the behaviour of mice in the plus-maze test

Mice exposed to small platform stress demonstrated an anxiolytic-like activity that was evidenced by an increase in the percentage of entries made onto and the percentage of time spent on the open arms. The total number of entries made onto the open and into the closed arms of the plus-maze was also increased (*Table 1, Table 2*).

In control mice flumazenil in a dose of 2.0 mg/kg did not have effect on the behaviour of mice in the plus-maze test. In a higher dose of 10.0 mg/kg, flumazenil decreased the percentage of entries made onto and the percentage of time spent on the open arms. In both doses used flumazenil did not influence the total number of entries made by control mice in the plus-maze test (*Table 1*).

Table 1. The effect of flumazenil, Ro 15-4513 and β-CCM on the behaviour of control and small platform stressed (SP) mice in the plus-maze test.

Drug, dose (mg/kg)	n	% of entries made onto the open arms		% of time spent on the open arms		Total number of entries made in the plus-maze test	
		Control	SP	Control	SP	Control	SP
Vehicle	14	35.9±4.8	46.2±2.9**	17.8±3.2	29.6±2.7***	19.6±0.8	24.5±1.3***
Flumazenil 2.0	6	27.0±1.3	13.1±2.2^{+++}	16.7±2.1	6.7±1.2^{+++}	19.5±0.9	18.5±2.1^{++}
10.0	6	18.9±1.6***	22.7±2.5^{+++}	6.2±0.7***	7.9±0.6^{+++}	17.5±0.7	14.5±2.1^{+++}
Ro 15-4513 1.0	6	15.6±2.1***	32.5±1.9^{++}	4.5±1.0***	13.4±1.2^{+++}	18.3±1.3	13.5±1.7^{+++}
3.0	6	6.3±1.5***	3.9±2.6^{+++}	1.1±0.3***	0.5±0.3^{+++}	15.5±0.5^{+}	1.5±1.0^{+++}
β-CCM 1.0	8	28.9±2.9	31.6±2.6^{+++}	15.6±1.0	14.1±1.6^{+++}	16.4±0.9	18.5±1.3^{+++}
2.0	8	24.3±4.4**	32.6±1.9^{+++}	9.8±1.2**	10.0±1.6^{+++}	11.9±1.6***	14.6±1.0^{+++}

* — $P < 0.05$, ** — $P < 0.01$, *** — $P < 0.001$ versus control/vehicle; $^{++}$ — $P < 0.01$, $^{+++}$ — $P < 0.001$ versus SP stressed/vehicle (Bonferroni test).

In small platform stressed mice flumazenil in both doses used (2.0 and 10.0 mg/kg) decreased the percentage of entries made onto the open arms, the percentage of time spent on the open arms and the total number of entries in the plus-maze test (*Table 1*).

Two way ANOVA revealed significant interaction between the effects of stress and flumazenil on the percentage of entries made onto the open arms

[F(2,46) = 4.493; $p < 0.05$], on the percentage of time [F(2,46) = 6.758; $p < 0.005$] and on the total number of entries [F(2,46) = 4.819; $p < 0.05$].

Ro 15-4513 and β-CCM in both control and small platform stressed mice dose-dependently decreased the percentage of entries made onto the open arms, the percentage of time spent on the open arms and the total number of entries (*Table 1, Table 2*).

Table 2. The effect of Ro 15-4513 on the behaviour of control and small platform stresed (SP) mice in the plus-maze test.

Drug, dose (mg/kg)	n	% of entries made onto the open arms		% of time spent on the open arms		Total number of entries made in the plus-maze test	
		Control	SP	Control	SP	Control	SP
Vehicle	6	28.2±5.0	34.0±3.2	16.9±3.8	25.1±3.9*	14.1± 2.4	20.3±3.4*
Ro 15-4513 0.5	6	27.5±5.8	29.7±7.0	15.9±2.8	16.4±3.4^{+}	15.8±2.3	13.1±3.3^{+}
1.0	6	13.7±6.1	24.1±5.2	7.5±4.2*	7.0±2.1^{++}	10.1±1.8	8.1±1.3^{+++}
2.5	6	10.8±5.2*	14.4±8.0^{+}	4.0±2.2***	1.1±0.5^{+++}	6.8±1.8*	4.6±1.5^{+++}
5.0	6	7.1±4.9*	7.5±4.8^{++}	2.0±1.6***	1.0±0.7^{+++}	5.5±2.0**	3.0±1.5^{+++}
10.0	8	7.5±4.8*	11.1±7.0^{++}	1.5±1.0***	0.4±0.3^{+++}	2.3±0.7***	2.0±0.3^{+++}

* — $P < 0.05$, *** — < 0.001 versus control/vehicle;
$^{+}$ — $P < 0.05$, $^{++}$ — $P < 0.01$, $^{+++}$ $P < 0.001$ versus SP stressed/vehicle (Bonferroni test).

DISCUSSION

The small platform technique, originally designed for the selective deprivation of REM sleep (16, 17) is currently considered a model of stress where rapid eye movement sleep deprivation is only one factor (18, 19). Although in our previous neurochemical experiments isolation, immobilization on large platforms (d = 9.0 cm) and repeated swimming stress had no influence on ^{3}H Flunitrazepam binding or GABA-stimulated $^{36}Cl^{-}$ uptake (22), it is probable that a combination of stress factors rather than one single factor might be responsible for the observed alterations in the activity of the $GABA_A$ receptor-chloride ionophore complex. Therefore, the results are discussed in the terms of stress, not in the terms of selective rapid eye movement sleep deprivation.

In accordance with our previous studies (21, 23) small platform stress significantly increased the percentage of entries made onto and the percentage of time spent on the open arms of the plus-maze, suggesting possible anxiolytic-like effect of small platform stress (26, 27). This effect was accom-

panied by an increase in the total number of entries. It may be supposed that an anxiolytic-like behaviour of small platform stressed mice in the plus-maze is solely due to the increased locomotions. However, it has been shown that anxiolytic drugs increase, whereas anxiogenic drugs decrease the total number of entries. Therefore it may be proposed that changes in the total number of entries in the plus-maze reflect the level of anxiety rather than changes in the locomotor activity (28).

In control mice flumazenil in a dose of 2.0 mg/kg had no effect and in a dose of 10.0 mg/kg exerted anxiogenic effect in the plus-maze test, as evidenced by a decrease in the percentage of entries made onto and the percentage of time spent on the open arms. These results are in confirmation with previous results where anxiogenic effect of flumazenil was seen after high doses (29). Although flumazenil was initially considered as a "neutral" benzodiazepine antagonist, lacking of an intrinsic activity (30), more recent data demonstrate that the effect of flumazenil is greatly dependent on the dose and the level of anxiety of the laboratory animals (31). In animals with high level of anxiety, flumazenil can produce anxiolytic-like effect, whereas in animals with low level of anxiety — an anxiogenic effect (32—34). This assumption is supported by the fact that the anxiolytic effect of flumazenil has been observed in humans in simulated stress (35). In our experiments small platform stress resulted in the enhancement of the anxiogenic effect of flumazenil in the plus-maze test. In small platform stressed mice the anxiogenic effect of flumazenil was seen at a dose of 2.0 mg/kg that was ineffective in control mice.

In agreement with previous studies (36, 37) the partial benzodiazepine receptor inverse agonist Ro 15-4513 and the full benzodiazepine receptor inverse agonist β-CCM exerted anxiogenic effect in the plus-maze test. The anxiogenic effect of these drugs was not affected by small platform stress exposure.

On the basis of our data it may be proposed that small platform stress increases the sensitivity of mice to the anxiogenic effect of flumazenil, but not β-CCM and Ro 15-4513.

There are several hypothesis about the selective enhancement of flumazenils action.

One possible explanation is that the weak anxiogenic effect of flumazenil is seen at a lower dose because of the anxiolytic-like effect of small platform stress. However, this assumption is contradicted by the fact that we were unable to show any enhancement of Ro 15-4513 effect even by using a wide range of doses (0.5—10.0 mg/kg).

The enhancement of flumazenil's action might also be explained with its antagonistic action at the benzodiazepine receptor. It is possible that small platform stress increases the concentration of the endogenous benzodiazepine

receptor ligand with stress protective activity. An existence of such a ligand has been proposed recently (2, 3). Such a proposal is in agreement with our behavioural studies demonstrating anxiolytic-like behaviour found in small platform stressed mice. Flumazenil may act by blocking the effects of this endogenous benzodiazepine receptor ligand. Another explanation might be, that observed in our previous studies increase in benzodiazepine receptors in small platform animals is a compensatory reaction to reduced GABAergic tone (22). The blockade of benzodiazepine receptors by flumazenil would unmask the reduced GABAergic tone and thereby increase the level of anxiety.

REFERENCES

1. Biggio G, Concas A, Corda MG, Giorgi O, Sanna E, Serra M. GABAergic and dopaminergic transmission in the rat cerebral cortex: effect of stress, anxiolytic and anxiogenic drugs. *Pharmac Ther* 1990; 48: 121—142.
2. Drugan RC, Paul SM, Crawley JN. Decreased forebrain [^{35}S]TBPS binding and increased [^{3}H]muscimol binding in rats that do not develop stress-induced behavioral depression. *Brain Res* 1993; 631: 270—276.
3. Drugan RC, Basile AS, Ha J-L, Ferland RJ. The protective effects of stress control may be mediated by increased brain levels of benzodiazepine receptor agonists. *Brain Res* 1994; 661: 127—136.
4. Boix F, Fernandez-Teruel A, Tobena A. The anxiolytic action of benzodiazepines is not present in handling-habituated rats. *Pharmacol Biochem Behav* 1989; 31: 541—546.
5. Conti LH, Maciver CR, Ferkany JW, Abreu ME. Footschock-induced freezing behavior in rats as a model of assessing anxiolytics. *Psychopharmacol* 1990; 102: 492—497.
6. Charrier D, Dangoumau L, Puech AJ, Hamon M, Thiebot M-H. Failure of CCK receptor ligands to modify anxiety-related behavioural suppression in an operant conflict paradigm in rats. *Psychopharmal* 1995; 121: 127—134.
7. Stone EA, Manavalan SJ, Zhang Y, Quatermain D. Beta adrenoceptor blockade mimics effects of stress on motor activity in mice. *Neuropsychopharmacol* 1995; 12: 65—71.
8. Ferretti C, Blengio M, Gamalero SR, Ghi P. Biochemical and behavioural changes induced by acute stress in a chronic variate stress model of depression: the effect of amitryptilline. *Eur J Pharmacol* 1995; 280: 19—26.
9. Andrews N, File SE. Handling history of rats modifies behavioural effects of drugs in the elevated plus-maze test of anxiety. *Eur J Pharmacol* 1993; 235: 109—112.
10. McBlane JW, Handley SL. Effects of two stressors on behaviour in the elevated X-maze: preliminary investigation of their interaction with 8-OH-DPAT. *Psychopharmacol* 1994, 116: 173—182.
11. Armario A, Gil M, Marti J, Pol O, Balasch J. Influence of various acute stressors on the activity of adult male rats in a hole-board and in the forced swim test. *Pharmacol Biochem Behav* 1991; 39: 373—377.
12. Willner P, Lappas S, Cheeta S, Muscat R. Reversal of stress-induced anhedonia by the dopamine receptor agonist, pramipexole. *Psychopharmacol* 1994; 115: 454—462.
13. Monleon S, D'Aquila P, Parra A, Simon VM, Brain PF, Willner P. Attenuation of sucrose consumption in mice by chronic stress and its restoration by imipramine. *Psyhopharmacol* 1995; 117: 453—457.

14. Moreau J-L, Scherschlicht R, Jenck F, Martin JR. Chronic mild stress-induced anhedonia model of depression: sleep abnormalities and curative effects of electroshock treatment. *Behav Pharmacol* 1995; 6: 682—687.
15. Cheeta S, Broekkamp C, Willner P. Stereospecific reversal of stress-induced anhedonia by mianserin and its (+)-enantiomer. *Psychopharmacol* 1994; 116: 523—528.
16. Morden B, Mitchell G, Dement W. Selective REM sleep deprivation and compensation phenomena in the rat. *Brain Res* 1967; 5: 339—349.
17. Mark J, Heiner L, Mandel P, Godin Y. Norepinephrine turnover in brain and stress reactions in rats during paradoxical sleep deprivation. *Life Sci* 1969; 8: 1085—1093.
18. Fratta W, Collu M, Marcelotta MC, Pichiri M, Muntoni F, Gessa GL. Stress induced insomnia: opioid-dopamine interactions. *Eur J Pharmacol* 1987; 142: 437—440.
19. Fratta W, Fadda P, Marcelotta MC, Gessa GL. Dopamine and neuropeptides interactions in sleep deprivation. *Clin Neuropharmacol* 1990; 13: 386—388.
20. Moore JD, Hayes C, Hicks RA. REM sleep deprivation increases preference for novelty in rats. *Physiol Behav* 1979; 23: 975—976.
21. Pokk P, Zharkovsky A. The effects of drugs acting at GABA-Benzodiazepine-Barbiturate receptor complex on the behaviour of sleep deprived mice. *Pharmacol Toxicol* 1995; 76: 23—28.
22. Pokk, P, Kivastik T, Sobol D, Liljequist S, Zharkovsky A. Is upregulation of benzodiazepine receptors a compensatory reaction to reduced GABAergic tone in the brain of stressed mice? *Naunyn-Schmied Arch Pharmacol* 1996; 354: 703—708.
23. Pokk P, Zharkovsky A. Ro 15-4513 potentiates, instead of antagonizes, ethanol-induced sleep in mice exposed to small platform stress. *Eur J Pharmacol* 1996; 317: 15—20.
24. Vale NB, Leite JR. Decreased susceptibility to local anesthetics-induced convulsions after paradoxical sleep deprivation. *Psychopharmacol* 1988; 94: 138—140.
25. Asakura W, Matsumoto K, Ohta H, Watanabe H. Involvement of dopamine D2 receptor mechanism in the REM sleep deprivation-induced increase in swimming activity in the forced swimming test. *Pharmacol Biochem Behav* 1994; 48: 43—46.
26. File SE, Wilks LJ, Mabbutt PS. Withdrawal, tolerance and sensitization after a single dose of lorazepam. *Pharmacol Biochem Behav* 1989; 31: 937—940.
27. Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacol* 1987; 92: 180—185.
28. Dawson GR, Crawford SP, Collinson N, Iversen SD, Tricklebank MD. Evidence that the anxiolytic-like effects of chlordiazepoxide on the elevated plus maze are confounded by increases in locomotor activity. *Psychopharmacol* 1995; 118: 316—323.
29. File SE. The anxiogenic action of benzodiazepine antagonists. *Neuropharmacol* 1982; 21: 1033—1037.
30. Crawley JN, Skolnick P, Paul SM. Absence of intrinsic antagonist actions of benzodiazepine antagonists on an exploratory model of anxiety in the mouse. *Neuropharmacol* 1984; 23: 531—537.
31. File SE, Pellow S. Intrinsic actions of the benzodiazepine receptor antagonist Ro 15-1788. *Psychopharmacol* 1986; 88: 1—11.
32. File SE, Baldwin HA, Hitchcott PK. Flumazenil but not nitrendipine reverses the increased anxiety during ethanol withdrawal in the rat. *Psychopharmacol* 1989; 98: 262—264.
33. Urbancic M, Gadek MA, Marczynski TJ. Chronic exposure to flumazenil: anxiolytic effect and increased exploratory behavior. *Pharmacol Biochem Behav* 1990; 35: 503—509.
34. Ferre P, Fernandez Teruel A, Escorihuela RM, Garcia E, Zapata A, Tobena A. Struggling and flumazenil effects in the swimming test are related to the level of anxiety in mice. *Neuropsychobiology* 1994; 29: 23—27.
35. Kapczinski F, Curran HV, Gray J, Lader M. Flumazenil has an anxiolytic effect in simulated stress. *Psychopharmacol* 1994; 114: 187—189.

36. Belzung C, Misslin R, Vogel E, Dodd RH, Chapouthier G. Anxiogenic effects of methyl-β-carboline-3-carboxylate in a light/dark choice situation. *Pharmacol Biochem Behav* 1987; 28: 29—33.
37. Nicholls B, Mellanby J, Smith S. The effect of Ro 15-4513, an inverse agonist at the benzodiazepine receptor, on the exploratory response to novelty in the playground maze. *J. Psychopharmacol* 1994; 8: 32—39.

R e c e i v e d: November 12, 1996
A c c e p t e d: April 4, 1997

Author's address: P. Pokk, Department of Pharmacology, University of Tartu, EE 2400 Tartu, Estonia