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Effects of diazepam and β-CCM on working memory in mice: relationships with emotional reactivity

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Abstract

This study was aimed at determining the effects of systemic administration of diazepam and methyl beta-carboline-3-carboxylate (β-CCM) both on spatial working memory and on emotional reactivity in mice. Results showed that diazepam and β-CCM induced opposite effects in both memory and emotional reactivity tests. Indeed, as a function of dose, diazepam reduced anxiogenic-like reactions but increased vulnerability to interference in the memory task at a 30-s but not at a 5-s delay interval. As a function of dose, β-CCM reduced vulnerability to interference and increased emotional reactivity, these effects being antagonised by concurrent administration of flumazenil (RO 15-1788). Thus, our study showed the bidirectional effects of these two drugs on a spatial working memory task involving a spontaneous processing of information and suggested a direct link between the emotional effects of the drugs and memory performance.

Keywords: Elevated plus maze; Open-field; Working memory; Proactive interference; Forgetting; Benzodiazepines; Flumazenil (RO 15-1788); Beta-carbolines

1. Introduction

Benzodiazepines (BDZ) are widely used anxiolytics and anticonvulsants that are also known for their sedative properties (Haefely, 1988). Certain BDZ have psychopharmacological properties that are opposite to those of agonist benzodiazepines. These substances, termed inverse agonists such as methyl beta-carboline-3-carboxylate (β-CCM) have proconvulsant effects and are anxiogenic (Belzung et al., 1987; Braestrup et al., 1980; Corda et al., 1983; File and Baldwin, 1987; Lister and File, 1987; Oakley and Jones, 1982).

Interestingly, in addition to these effects, it has been shown that anterograde amnesia may be observed following the administration of BDZ and/or GABA agonists or antagonists, such as faster forgetting rates and/or exaggerated vulnerability to interference (Chapouthier et al., 1991; Chrobak and Napier, 1992; Lister, 1985; Stackman and Walsh, 1992; Venault et al., 1986). Several studies have also shown that BDZ agonists and inverse agonists bidirectionally modulate performance in several learning and memory tasks (impairment or improvement, respectively) both in humans and animals (Chapouthier and Martin, 1992; Jensen et al., 1987; Venault et al., 1986). Interestingly, it has been already demonstrated in humans that the effects of BDZ on memory were also dependent on the processing (automatic vs. effortful) of information and/or the type of memory (Danion et al., 1993; Danion et al., 1989; File et al., 1992).

In line with this idea, acquisition deficits have been shown in tasks involving a strong procedural component (Borde and Beracochea, 1999; Kesner, 1986; Morris, 1983; Venault et al., 1986) but, once acquired, these tasks were relatively insensitive to the effects of BDZ (Belzung et al., 1987; Lister and File, 1987). In contrast, working memory deficits have been reported in animals administered either with BDZ agonists or with compounds acting on the GABA receptor (Borde et al., 1998; Chrobak and Napier, 1992; Sarter, 1990; Stackman and Walsh, 1992). However, the tasks used in these animal studies involved food

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deprivation, the use of a positive or negative reinforcement, as well as extensive prior training procedures that engaged the subjects in goal-oriented behaviour; so far, these working memory tasks involved to some extent an active processing of information. In contrast, studies of the effects of BDZ on a working memory task involving a more spontaneous processing of information are scarce. It is thus of interest to determine if the bidirectional memory effects of benzodiazepines agonists and inverse agonists already reported in animals studies are also observed in a memory task involving more spontaneous memory processing. Thus, the first goal of our study was to determine whether diazepam and β-CCM, respectively, would induce opposite memory effects in a nonrewarded spontaneous alternation (SA) task involving spatial working memory and which is based on an innate (as opposed to a learned and reinforced) behavioural rule (Bracocha and Jaffard, 1987; Bracocha and Jaffard, 1990; Divac et al., 1975; Douglas, 1967; Grant, 1981; McNaughton and Feldon, 1980; Olton and Samuelson, 1976; Roberts and Dale, 1981; Thomas, 1984). Indeed, SA does not require the use of any reinforcement or training to emerge, in so far as it is based on an innate tendency to explore a spatial environment, or to visit the last recently experienced spatial location. Thus, alternation at a given N trial is based on the relative strength of the memory trace as compared to the information received on trial $N-1$. Thus, alternation behaviour involves, in a nonrewarded procedure, only a spontaneous processing of information, and/or assesses a lower level of effortful working memory, as compared to most of the tasks currently used in animal studies.

Since diazepam and β-CCM induced, respectively, anxiolytic and anxiogenic effects, the second goal of our study was to determine if relationships existed between the effects of these compounds on memory and on emotional reactivity. The emotional state of a subject is influenced by BDZ administration (Gray, 1982) and it could interact with alternation performance. Indeed, in our alternation protocol, once the subject entered a goal-arm of the maze, the door to that arm was closed. Direct observation revealed that enclosing a mouse in a goal-arm induced mild fear reactions. Thus, the alternation behaviour at a given trial N seemed to be, at least in part, based on the tendency to avoid the last "negatively" experienced $(N-1)$ arm. This emotional component might help the subject to retain in memory the last arm visited. Thus, in line with the goals of our study, the SA task appears to be adequate to examine the eventual interaction between memory and the emotional state of a subject. In this case, if this is true, drugs acting on emotional reactivity should modulate (decrease or increase) alternation performance. In order to determine the eventual interaction between the memory and the emotional effects of the drugs, subjects were submitted to tests of anxiety (the open-field and elevated plus maze) known to be sensitive to BDZ administration (Dennenberg, 1969; File and Baldwin, 1987; Pellow et al., 1985), before being submitted to the alternation task. This successive testing procedure allows to conduct a correlation analysis between the anxiety and alternation scores. In order to determine the possible influence of one test on the other in this successive testing procedure, as shown in other studies (Rodgers and Cole, 1993), the effects of the drugs on elevated plus maze and alternation tasks were also determined using an independent testing procedure.

2. Materials and methods

2.1. Animals

The study was conducted using male mice of the Balb/c strain of 6 weeks of age from Iffa-Credo, Lyon (France) on arrival in the laboratory. Mice were initially housed collectively in colony cages ($40 \times 25 \times 20$ cm, length × width × height), matched for weight, and placed in an animal room (ambient temperature: 22°C; automatic light cycle: 08:00; 20:00 h) with free access to food and water. At least 2 weeks before behavioural testing began, mice were housed in individual cages, with free access to food and water.

2.2. Drugs

β-CCM was synthesised at the Institut de Chimie des Substances Naturelles (Gif-sur-Yvette, France). This compound was first dissolved in 0.1 N HCl and then diluted to final concentration with saline. Diazepam was diluted with saline. Flumazenil (Ro 15-1788), a selective BDZ antagonist (Haefely, 1988; Hunkeler et al., 1981), was suspended in saline solution with a drop of Tween 80 and homogenised. Drugs were administered subcutaneously (sc) or intraperitoneally (ip) in a volume of 0.05 ml/10 g (body weight).

2.3. Behavioural procedures

2.3.1. Sequential alternation

Working memory was assessed by examining SA behaviour. SA is the innate tendency of rodents to alternate on each trial, over a series of successive runs in a T-maze, the choice of the visited goal-arm. This procedure has been widely used as a behavioural tool to study rates of forgetting and/or sensitivity to proactive interference between spatial information. Sequential procedures enable to distinguish between the ability to alternate per se (second trials of a series) from the progressive inability to do so as the number of trials increases. In this latter case, repetitive testing (i.e., sequences of more than two trials) constitutes a potent source of proactive interference since the response on trial N (which depends on information received on trial $N-1$) may be influenced by all previous information received from Trial 1 to $N-2$. Accordingly, this task involves a strong working memory component, since a correct choice requires the subject to refer to a specific information, which

varies from trial to trial (Bracocha and Jaffard, 1987; McNaughton and Feldon, 1980; Thomas, 1984).

The task was performed in a T-maze constructed of grey Plexiglas (30 cm long, 10 cm wide and 25 cm high). All subjects were submitted to daily sessions of nine successive trials (sequential procedure) separated either by a 30-s intertrial interval (ITI, six first trials) or by a 5-s ITI (three last trials of the series). In such a procedure, using the same number of trials per session, we demonstrated a selective memory impairment following either lesions of several diencephalic and hypothalamic brain areas (Bracocha and Jaffard, 1987; Bracocha and Jaffard, 1990) or following a chronic alcohol treatment (Bracocha et al., 1987). Thus, the alternation procedure as used in the present study enables to detect memory effects of the drugs. In our procedure, the trials separated by the 5-s interval were not aimed at measuring working memory at short intervals, but rather to detect any possible sedative effects of the drugs on behavioural performance on the preceding 30-s trials. For this purpose, these 5-s trials were placed at the end of the session, to ensure that both the motivation to alternate and neurobiological processes sustaining the alternation response function normally. To begin a trial, the subject was placed in the starting-box (10×12 cm) for 30 s (six first trials) or 5 s ITI (three last trials). Once this time had elapsed, the door to the stem was opened. When the mouse entered one of the arms, the door to that arm was closed. Mice remained in the goal-arm for 30 s. Once this time had elapsed, the mouse was subsequently removed, and placed in the start-box for a new trial.

2.3.2. Open-field

The open-field was constructed of wood in the shape of a square measuring 120 cm per side. The floor was painted white and divided in 64 squares by black lines drawn on the floor. Illumination was provided by two lamps positioned 2 m above the apparatus and providing a 600-lx illumination equally distributed over the whole surface of the apparatus. At the start of each trial, animals were also placed on the centre of the apparatus in a small cylinder. Following a 30-s delay, the cylinder was removed and the subjects were allowed to freely explore the apparatus for 10 min. Two measures were taken. Firstly, the total number of squares crossed by the subjects. This measure was taken as an index of activity. Secondly, the number of fecal boli recovered after the 10-min period was used as an index of emotionality (Dennenberg, 1969).

2.3.3. Elevated plus-maze

The plus-maze, which was constructed of grey Plexiglas, consisted of four arms arranged in the shape of a plus sign. Each arm was 30 cm long, 7 cm wide and elevated 40 cm above the ground. The four arms were joined at the centre by a 7-cm square platform. Two opposite arms of the plus maze were ''closed'' by side walls 17 cm high, but open on the top, while the remaining arms did not have side walls. These walls did not extend from the centre of the maze. At the beginning of each test, mice were placed on the centre of the maze in a cylinder (8 cm diameter, 17 cm high) for 30 s. Then, the cylinder was removed and mice were allowed to freely explore all arms of the maze for 10 min. Activity and latencies were measured by the experimenter using semiautomatic counters and timers. An entry was counted only when a mouse entered an arm with all four paws. Two measures of ''anxiety'' were taken. The first was the ratio of the time spent in the open arms divided by the total time spent in all arms of the maze (time ratio). The second was the ratio of entries into the open arms divided by the total number of entries in all arms (entry ratio). Thus, the smaller are these ratios, the more ''anxious'' is the mouse (File and Baldwin, 1987; Pellow et al., 1985).

2.4. Experimental design

Independent groups of mice were injected with either saline (sc or ip), β-CCM (0.25, 0.5 and 1.0 mg/kg sc), or diazepam (1, 1.5 and 2 mg/kg ip). Flumazenil (15 mg/kg) was injected intraperitoneally either alone or in combination with β-CCM (0.5 mg/kg). In the latter case, flumazenil was injected 5 min before injection of β-CCM. Either 15 (flumazenil), 20 (saline, β-CCM) or 30 min (saline, diazepam) later, mice were submitted to behavioural testing.

Two different behavioural schedules were used:

First experiment: successive testing. Each subject was first submitted to the open-field test, then to the elevated plus-maze test followed by the SA task. The three tasks were run within a single period of 40 min. The open-field, elevated plus maze and SA tasks were each run in different rooms. Each mouse was tested only once in each task. Animals were not food deprived during the experiments. Two days before being submitted to behavioural testing and drug administration procedures, as described above, animals were given two free exploration sessions of the T-maze (one daily session) of 10 min each, in order to familiarise them with the apparatus.

Second experiment: independent testing. Since the alternation task was placed last in the task sequence of the first experiment, it was possible that previous anxiety testing could interact with subsequent alternation performance. Thus, the eventual bidirectional effect of the drugs on alternation performance could possibly reflect a differential tasks interaction as a function of the treatment, rather than an intrinsic effects of the drugs. We therefore also studied the effects of either β-CCM (0.5 mg/kg) or diazepam (2.0 mg/kg) administration in subjects performing only the alternation task or the elevated plus maze task. The doses used were those that induced a clear-cut memory effect in the first experiment. For each of these tasks, the behavioural procedure was similar to the one described in the first experiment.

2.5. Ethical statement

All pharmacological and experimental procedures were in accordance with official French Regulations for the Care and Use of Laboratory Animals.

2.6. Statistical analysis

Results were analysed on transformed data (arcsin values). The arcsin transformation was used to normalise raw data. The homogeneity of variance of the transformed data was verified with Bartlett's and Lenvene's statistical tests. Differences between groups were analysed using post-hoc factorial ANOVA.

3. Results

3.1. First experiment: successive testing

3.1.1. Tests of emotional reactivity

3.1.1.1. Global analysis. This analysis was performed using all groups of the first experiment (nine groups: controls, three diazepam groups, three β-CCM groups, one flumazenil group and one flumazenil + β-CCM group). Since no statistical significant differences were observed between the performance of the two saline-treated groups (intraperitoneal vs. subcutaneous) for any of the behavioural measures ($F < 1.0$ in all cases), these two groups were pooled (controls; $N = 42$) for further between-groups comparisons.

3.1.1.2. Open-field. ANOVAs revealed that there were significant effects of treatment on both activity and number of fecal boli [F's(8,130) > 4.6; $P < .001$]. Compared to controls, the largest changes were observed with diazepam (1.5 mg/kg) producing a significant increase in activity (from 50.4 to 117; $P < .001$) and a significant decrease in the number of fecal boli (from 5.5 to 0.0; $P < .001$; see Table 1), and with β-CCM at 1 mg/kg that produced an inverse pattern of changes (i.e., a nonsignificant decrease in activity from 50.4 to 36.7 and a significant increase in the number of fecal boli (from 5.5 to 8.4; $P < .001$; see Table 2).

3.1.1.3. Elevated plus maze. ANOVAs revealed that there was a significant effect of treatments on both entry ratio and time ratio [F's(8,130) > 19.5; $P < .001$] whereas the total number of entries were not significantly different among groups [$F(8,130) = 1.74$; $P > .05$]. Compared to controls, the largest changes were again observed with diazepam at 1.5 mg/kg, which significantly increased both ratios (from .34 to .59 and .34 to .82; $P < .001$ for both comparisons; see Table 1) and with β-CCM, which produced an inverse pattern of changes (i.e., a significant decrease in both entry and time ratios: respectively, from .34 to .14 and .34 to .15; $P < .001$ in both cases; see Table 2).

3.1.2. Effects of diazepam

3.1.2.1. Open-field. Results are summarised in Table 1 (left). Compared to saline, diazepam administration resulted in a highly significant reduction in the number of fecal boli (three groups treated with diazepam + control group; $F(3,81) = 43.5$; $P < .001$), together with an increase in activity [$F(3,81) = 7.5$; $P < .001$]. This increase in activity was significantly higher at 1.5 mg/kg than at 2 mg/kg ($P < .05$).

3.1.2.2. Elevated plus-maze. Results are summarised in Table 1 (right). Injection of diazepam increased both the entry ratio [Groups; $F(3,81) = 12.1$; $P < .001$] and time ratio [$F(3,81) = 37.1$; $P < .001$] but an absence of a dose-dependence was observed ($P > .20$) Even though the total number of entries was not significantly different among groups [$F(3,81) = 1.9$; $P = .13$] it can be noted that administration of the lowest dose of diazepam (1.0 mg/kg) produced a significantly higher number of total entries than the 2.0 mg/kg dose; ($P < .05$).

3.1.3. Effects of β-CCM

3.1.3.1. Open-field. Results are summarised in Table 2 (left). A highly significant between-group difference was observed in the number of fecal boli [three groups treated

Table 1
Measures of activity and fecal boli (means ± S.E.M.) in the open-field task (left) and entries and time ratios (means ± S.E.M.) observed in the elevated plus-maze (right) in saline- and diazepam-treated subjects (Diaz.)

	Open-field		Elevated plus-maze		
Groups (N)	Activity	Fecal boli	Entry ratio	Time ratio	Total entries
Saline (42)	50 ± 5.5	5.5 ± 0.4	0.34 ± 0.03	0.34 ± 0.03	21.0 ± 1.7
Diaz. 1.0 (10)	87.2 ± 22.2*	0.8 ± 0.4***	0.55 ± 0.04***	0.79 ± 0.04***	27.3 ± 3.4*
Diaz. 1.5 (10)	117 ± 17***	0.0***	0.59 ± 0.04***	0.82 ± 0.03***	22.4 ± 3.8
Diaz. 2.0 (20)	78.4 ± 8.4*	0.4 ± 0.2***	0.54 ± 0.03***	0.72 ± 0.04***	17.9 ± 1.7

Diazepam concentrations are expressed in mg/kg. N = number of subjects per group.
* Significantly different from saline $P < .05$.
** $P < .01$ compared to saline-treated mice.
*** Significantly different from saline $P < .001$.

Table 2
Measures of activity and fecal boli (means ± S.E.M.) in the open-field task (left) and measures of the entry and time ratios (means ± S.E.M.) in the elevated plus-maze (right) in saline- and β-CCM-treated subjects

	Open-field		Elevated plus-maze		
Groups (*N*)	Activity	Fecal boli	Entry ratio	Time ratio	Total entries
Saline (42)	50.4 ± 5.5	5.5 ± 0.4	0.34 ± 0.03	0.34 ± 0.03	21.0 ± 1.7
β-CCM 0.25 (8)	43.0 ± 11.0	0.6 ± 0.3***	0.51 ± 0.06**	0.56 ± 0.06***	17.5 ± 1.8
β-CCM 0.5 (15)	47.5 ± 10.7	7.8 ± 0.5***	0.17 ± 0.01***	0.15 ± 0.02***	18.5 ± 1.7
β-CCM 1.0 (14)	36.7 ± 9.9	8.4 ± 0.7***	0.14 ± 0.02***	0.15 ± 0.02***	18.4 ± 1.7
Ro 5-1788 (7)	64.7 ± 24.4	1.1 ± 0.9***	0.45 ± 0.02	0.57 ± 0.03***	22.3 ± 1.0
Ro 5-1788 + β-CCM 0.5 (13)	43.4 ± 9.1	3.0 ± 0.7***	0.31 ± 0.03	0.29 ± 0.06	16.2 ± 1.3

Drugs concentrations are expressed in mg/kg. *N* = number of subjects per group.
* $P < .05$ compared to saline.
** Significantly different from saline $P < .01$.
*** Significantly different from saline $P < .001$.

with β-CCM + control group: $F(3,78) = 20.6$; $P < .001$]. However, depending on the dose administered, β-CCM produced opposite effects with respect to the control group. Thus, whereas the two highest doses used (0.5 and 1 mg/kg) significantly increased the number of fecal boli ($P < .001$ for both comparisons with controls), the lowest dose (0.25 mg/kg) actually resulted in a highly significant decrease in the number of fecal boli ($P < .001$ compared to controls). The mean level of activity was not significantly different among groups ($F < 1.0$).

3.1.3.2. Elevated plus-maze. Results are summarised in Table 2 (right). A highly significant between-group difference was observed on both entry ratio [$F(3,78) = 15.3$; $P < .001$] and time ratio [$F(3,78) = 15.5$; $P < .001$]. Again, whereas at the doses of 0.5 and 1 mg/kg both indices were significantly decreased relative to controls ($P < .001$ for all four comparisons), the lowest dose (0.25 mg/kg) induced an opposite pattern of changes ($P < .01$ as compared to controls). Total entries were not significantly different among groups ($F < 1$).

3.1.4. Effects of flumazenil on the emotional effects of β-CCM

3.1.4.1. Open-field. Results are summarised in Table 2 (bottom). As a whole, flumazenil (RO 15-1788; 15 mg/kg) decreased indices of anxiety as measured by both a significant decrease in fecal boli ($P < .001$) in the open-field, and a significant increase in time ratio ($P < .001$) in the plus-maze as compared to controls. Flumazenil partially antagonised the anxiogenic effects of β-CCM at the dose of 0.5 mg/kg. Indeed, when β-CCM was administered in combination with flumazenil, there was a clear tendency of anxiety scores to normalise with respect to the control group ($P > .05$ for all comparisons; see Table 2). Moreover, as compared to the group receiving β-CCM alone, animals injected with both drugs displayed a significant decrease in fecal boli in the open-field ($P < .001$) together with a significant increase in entry ratios and time ratios ($P < .05$ for both indices).

3.1.5. T-maze alternation

3.1.5.1. Global analysis. This analysis was performed using all groups of the first experiment. ANOVA revealed that over the entire session (nine trials), there was a significant effect of treatments on SA rates [$F(8,114) = 5.16$; $P < .001$]. As compared to controls, the largest changes were observed with diazepam at 2 mg/kg inducing a significant decrease in SA rates (from 73.7% to 53.7%; $P < .001$) and with β-CCM at 0.5 mg/kg that, inversely, tended to induce a slight but nonsignificant increase in SA rates (73.7% to 81.3%; $P > .05$). Interestingly, when successive blocks of trials were considered separately, ANOVAs revealed that the only significant between-group differences were observed on Trials 4–6 (30 s ITI) [$F(8,114) = 4.45$; $P < .001$], whereas no significant differences were observed on Trials 2–3 (30 s ITI; $P > .20$) and 7–9 (5 s ITI; $P > .80$) (see also Figs. 1 and 2). This suggests that the observed drug-induced alterations of SA depends both on the ITI (i.e., 30 s vs. 5 s) and, with the 30 s ITI, on the position of trials within the series (i.e., 2–3 vs. 4–6).

3.1.6. Effects of diazepam

Results are summarised in Fig. 1

3.1.6.1. Trials 2–6 (30 s ITI). A significant between-groups difference was observed [three groups treated with diazepam + control group: $F(3,57) = 6.11$; $P = .001$]. Post-hoc paired comparisons following an ANOVA on all groups showed that at the dose of 2 mg/kg diazepam significantly reduced SA rates as compared to controls (42.0% vs. 72.9%; $P < .01$). Lower doses (1 and 1.5 mg/kg) also decreased SA rates (58% for both doses; $P < .05$ for both comparisons). However, as shown in Fig. 1, animals injected with the lowest dose of diazepam (1 mg/kg), actually displayed normal SA rates on the first pooled two trials (trials 2 + 3: 75.0% vs. 72.6% for controls), but were significantly impaired on the pooled last three trials (trials 4–6: 46.6% vs. 73.2%; $P < .05$) [interaction block of trials × treatment: $F(1,39) = 3.67$; $P = .06$].

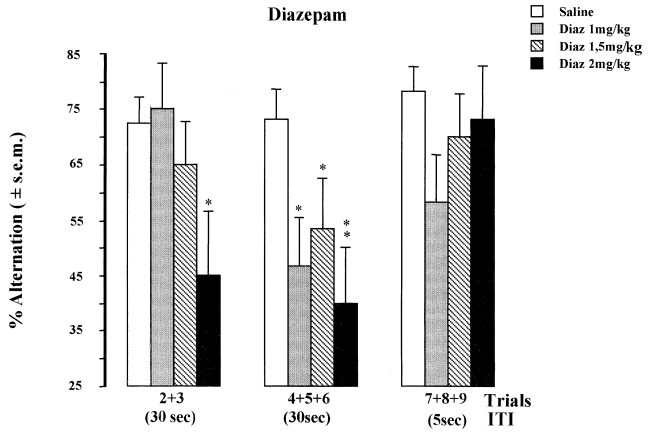

Fig. 1. Mean % SA (±S.E.M.) as a function of the position of the trial within the series; * $P<.05$; ** $P<.01$ compared to saline-treated mice.

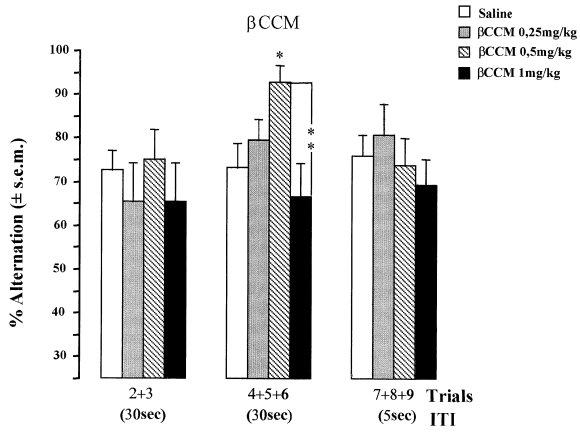

Fig. 2. Mean % SA (±S.E.M.) as a function of the position of the trial within the series; * $P<.05$ compared to saline-treated mice; ** $P<.01$ compared to β-CCM 0.5 mg/kg.

3.1.6.2. Trials 7–9 (5 s ITI). No significant between-groups differences were observed [$F(3,47)=1.37$; $P=.26$].

3.1.6.3. Comparison of Trials 2–6 (30 s ITI) with Trials 7–9 (5 s ITI). As suggested by the above analysis, reducing the ITI from 30 (Trials 2–6) to 5 s (Trials 7–9) resulted in an overall increase in SA rate in diazepam-treated subjects (see Fig. 1). In particular, one can note that the 2 mg/kg group which, as compared to controls, was the most impaired across Trials 1 to 6 with a 30-s ITI ($P<.01$; see above), displayed normal SA rates on the last three trials with a 5-s ITI (73.3% vs. 76.0% for controls). However, (1) the interaction between performance recorded on the first block (2–6; 30 s ITI) and the last block of trials (7–9; 5 s ITI) and treatment did not reach statistical significance [$F(1,37)=3.27$; $P=.08$]; (2) in either case, these differences may be due either to the reduction of the ITI (from 30 to 5 s), or to the position of trials in the series (Trials 2–6 vs. 7–9). The following additional experiment was designed to determine which of these two factors was responsible for the differential effect of diazepam on SA rates.

3.1.6.4. Comparison of Trials 2–6 (5 s ITI) with Trials 7–9 (30 s ITI). In this experiment, the procedure was exactly the same as in the previous one, except that the ITI conditions were reversed with respect to the position of trials within the series [i.e., the ITI was increased from 5 (Trials 2–6) to 30 s (Trials 7–9)]. Results are summarised in Table 3. Compared to saline, diazepam (2 mg/kg) produced an overall significant decrease in SA rates [treatment: $F(1,18)=8.6$; $P<.01$]. As in the previous experiment, animals treated with diazepam at 2 mg/kg were not significantly different from controls with the 5 s ITI ($P>.10$) but were significantly impaired with the 30 s ITI [$F(1,18)=12.9$; $P<.01$]. However, the ITI (block) × treatment interaction again did not reach statistical significance [$F(1,18)=3.16$; $P=.09$]. Together with results from the previous experiment, this nevertheless suggests that the deleterious effect of diazepam at 2 mg/kg on SA was delay-dependent, as revealed by the significant treatment × ITI interaction found by pooling results from both experiments [$F(1,39)=5.54$; $P=.02$] (for further details, see Table 3).

3.1.7. Effects of β-CCM

Results are summarised in Fig. 2.

3.1.7.1. Trials 1–6 (30 s ITI). An ANOVA revealed that, overall (Trials 2–9), there was a significant effect of treatments [three groups treated with β-CCM + control group: $F(3,67)=2.98$; $P<.05$]. As can be seen in Fig. 2, this was due to differences in SA rates recorded on Trials 4–6 [Group: $F(3,67)=2.87$; $P<.05$] but not on Trials 2–3 ($F<1.0$). Post-hoc paired comparisons following ANOVA on all groups

Table 3
Mean % SA (±S.E.M.) as a function of ITI in the first, second and pooled experiments

	1st experiment		2nd experiment		Pooled	
ITI	30 s	5 s	5 s	30 s	5 s	30 s
Saline	74.3±9.4	74.5±5.5	82.0±3.6	83.4±5.6	78.2±5.1	78.7±3.9
Diazepam (2 m/kg)	42.0±7.6**	73.3±9.7	72.0±5.3	50.0±7.5**	72.7±5.4	46.0±5.3*

* Significantly different from saline $P<.05$.
** Significantly different from saline $P<.01$.
*** Significantly different from saline; $P<.001$.

showed that, on Trials 4–6, only β-CCM at 0.5 mg/kg significantly improved SA rate (92.9% vs. 73.2% for controls; $P<.05$), whereas the dose of 1 mg/kg actually produced a slight, but nonsignificant, decrease in SA rates (66.7%).

3.1.7.2. Trials 7–9 (5 s ITI). No significant between-group differences were observed ($P>.05$).

3.1.8. Effects of flumazenil on β-CCM-induced facilitation of SA

Results are summarised in Table 4. Overall (Trials 2–9), flumazenil (RO 15-1788; 15 mg/kg) produced a slight nonsignificant decrease in SA rates. Although not statistically significant, this decrease was mainly observed on Trials 4–6 with the 30 s ITI (52.5% vs. 73.2% for controls), whereas on both Trials 2–3 (30 s ITI) and 7–9 (5 s ITI) SA rates were close to control levels (see Table 4). Finally, the previously described increase in SA rate on Trials 4–6 (30 s ITI) produced by β-CCM at 0.5 mg/kg (from 73.2% to 92.9%) was totally antagonised by the co-administration of flumazenil (β-CCM + flumazenil: 60.1% vs. β-CCM: 92.9%; $P<.01$).

3.2. Correlation between tests of emotional reactivity and SA performance

Since, as a whole, diazepam and β-CCM have opposite effects on both anxiety and T-maze alternation, the question arises as to whether there would be a link between anxiety and alternation scores. Correlation between anxiety and alternation scores was established (Pearson correlation) followed by appropriate significance test. Results obtained for the entire population of subjects ($N=122$) are summarised in Table 5. Highly significant correlation ($P<.001$) were observed between indices of anxiety recorded in the open-field (fecal boli; $r=+.37$) and elevated plus-maze (time ratio: $r=-.39$), and SA rates recorded over the entire session (Trials 2–9); although significant ($P<.05$), the correlation between SA rates and entry ratios (elevated plus maze) was weaker ($r=-.26$); no correlation ($P>.40$) was observed between SA rates and activity in the open-field. Finally, as can be seen in Table 5, significant correlation were specifically linked to SA rates on Trials 4–6 (30 s ITI) but not on Trials 2–3 (30 s) and 7–9 (5 s).

Table 4
Mean % SA (±S.E.M.) as a function of the position of the trial within the series

Groups (*N*)	Trials 2–3	Trials 4–6	Trials 7–9
Saline (31)	72.6±4.5	73.2±5.5	76.0±4.9
β-CCM 0.5 (14)	75.0±6.9	92.9±3.8	73.9±6.2
RO 15-1788 (7)	78.6±10.1	52.5±10.0	71.4±10.1
RO 15-1788+β-CCM 0.5 (15)	66.7±6.3	60.1±9.3**	73.3±9.6

N=number of subjects per group.
** Significantly different from β-CCM 0.5 mg/kg; $P<.01$.

Table 5
Correlation between tests of emotional reactivity and SA as a function of ITI and the position of the trial within the series

	T-maze alternation			
ITI	30 s		5 s	
Trials	2–3	4–6	7–9	Mean
Open field				
Activity	+0.07	−0.13	−0.02	−0.07
Fecal boli	+0.11	+0.31***	+0.11	+0.37***
Plus-maze				
Entry ratio	−0.06	−0.28**	+0.01	−0.26*
Time ratio	0.00	−0.40***	−0.11	−0.39***

* $P<.05$.
** $P<.01$.
*** $P<.001$.

3.3. Second experiment: independent testing

This experiment was designed to determine whether previous tests of anxiety could interact with drug effects on alternation performance. Accordingly, this experiment was performed using the lowest dose of β-CCM (0.5 mg/kg) and the 2.0 mg/kg dose for the diazepam-treated group, these doses have shown a significant effect on alternation rates and emotional reactivity in the first experiment. Treated subjects were compared to their respective controls receiving either saline subcutaneously or intraperitoneally. Since no statistical differences were observed between the two control groups in all comparisons, they were pooled for further statistical comparisons with treated animals. Seventy animals were used in this study, and assigned to either the elevated plus maze task (β-CCM: $N=10$; diazepam: $N=10$; controls: $N=15$) or the alternation task (β-CCM: $N=10$; diazepam: $N=10$; controls: $N=15$). The behavioural procedures for the elevated plus maze and the alternation tasks were similar to the ones described in the successive testing schedule.

3.4. Elevated plus maze

An analysis performed on the three groups (β-CCM, diazepam and controls) showed significant differences both for the entry ratio [$F(2,34)=30.6$; $P<.001$] and for the latency ratio [$F(2,34)=52.9$; $P<.001$]. More specifically, diazepam significantly increased both the entry ratio as compared to controls (0.5±0.03 vs. 0.37±0.03, respectively; $P<.001$) and the latency ratio (0.74±0.04 vs. 0.39±0.04 for diazepam and controls, respectively); in contrast, β-CCM significantly reduced both the entry ratio (0.19±0.03) and the latency ratio (0.17±0.02) as compared to controls ($P<.001$ in each case). Interestingly, no significant differences ($F<1.0$) were observed between subjects of the first experiment (successive testing procedure) and of the second experiment (independent testing procedure) in each group, suggesting that previous exposure to

the open-field did not modify performance on the elevated plus maze in the successive testing procedure.

3.5. T-maze alternation

3.5.1. Trials 2–6 (30 s ITI)

A significant between-groups difference (three groups: β-CCM, diazepam and controls) was observed [$F(2,34) = 5.12$; $P < .01$]. Post-hoc paired comparisons showed that diazepam significantly reduced SA rates as compared to controls [$48.0 \pm 1.3\%$ vs. $74.6 \pm 4.9\%$, respectively; $F(1,24) = 8.9$; $P < .006$]. Whereas no between groups differences were observed on the first block of trials (Trials 2–3; $F < 1.0$), a significant difference was observed on the second block of trials (Trials 4–6) [$77.8 \pm 7.7\%$ vs. $38.6 \pm 3.1\%$ for controls and diazepam-treated subjects, respectively; $F(1,24) = 9.9$; $P < .004$]. In contrast, β-CCM significantly increased alternation rates as compared to controls [$90.5 \pm 3.8\%$; $F(1,24) = 5.5$; $P < .03$], and this effect was mainly observed at the second block of trials (94.3 ± 2.1; $P < .03$ as compared to controls). Interestingly, comparisons of performance in diazepam-treated (2.0 mg/kg) subjects on alternation performance of the first and second experiments showed nonsignificant between groups difference ($P > .05$), this being also the case for β-CCM-treated (0.5 mg/kg) and control subjects ($P > .05$ in each comparison). Thus, pre-exposure to anxiety testing had no significant effect on performance in the alternation task, and did not interact with the intrinsic effects of the drugs.

3.5.2. Trials 7–9 (5 s ITI)

No behavioural differences were observed between the different groups (77.6 ± 4.5; 82.5 ± 3.4 and 87.5 ± 5.2 for diazepam, controls and β-CCM groups, respectively; $P > .05$).

4. Discussion

The aims of this study were (i) to compare the effects of systemic administration of diazepam and β-CCM on both emotional reactivity in anxiogenic tests and a spontaneous form of working memory in a T-maze alternation (SA) task and (ii) to determine whether the observed changes in SA rates may be correlated to the emotional effects of the drugs.

4.1. Effects on memory

With a 30 s ITI, diazepam induced an overall (Trials 2–6) dose-dependent deficit in SA. However, with the lowest dose (1 mg/kg; see Fig. 1), SA rates were strongly reduced on the three last (Trials 4–6) but not on the first two trials of the series (Trials 2–6). This would suggest that diazepam induces an increased sensitivity to proactive interference. An alternative hypothesis, however, is that such a decrease would stem from changes in exploratory tendencies across successive trials, leading thereby to a progressive loss of the motivation to alternate. This possibility is congruent with the observation that, over the three last trials of the series (i.e., Trials 7–9), animals treated with diazepam at 1 mg/kg did not completely recover despite a reduction of the ITI from 30 to 5 s, the normal SA rates that they exhibited on the two first trials of the series (i.e., Trials 2–3). Notwithstanding, it seems difficult to reconcile this interpretation with results obtained with the highest doses of diazepam (2 mg/kg). Indeed, at this dose (and to a lesser extent at the dose of 1.5 mg/kg), it appeared that animals completely recovered normal SA rates on Trials 7–9, as soon as the ITI was shifted from 30 to 5 s. In this case, the possibility that a recovery might result from a change in the "context" of testing (i.e., a shift in the ITI) rather than to the use of a short (5 s) ITI per se, seems to be ruled out given that in the reverse conditions (i.e., 5 s on the first five trials and 30 s on the last three one), the performance of animals treated with diazepam at 2 mg/kg dropped to chance level when the ITI was shifted to 30 s. Taken together, these results suggest that the observed diazepam-induced alterations in SA rates result from alterations in mnemonic processes that sustain alternation behaviour and, more widely, spatial working memory. More precisely, given the delay-dependent deleterious effects of diazepam on SA behaviour, it may be postulated that part of this deficit may be explained by faster forgetting notably (if not exclusively) in testing conditions that increase levels of proactive interference. The recovery of SA behaviour on Trials 7 to 9 as a function of the ITI also enables us to discard possible sedative effects of the drugs or impairment of the behavioural inhibition system as possible factors responsible for the deficits, as has already been shown with other BDZ drugs (Chapouthier and Martin, 1992).

As a whole, as compared to diazepam, β-CCM administration produced an opposite pattern of results on SA rates (see Fig. 2) to those observed with diazepam. As concerns the only dose of β-CCM that produced a significant improvement of SA rates (i.e., 0.5 mg/kg), it again appeared that this enhancing-memory effect was not uniform across the series of trials but specific to Trials 4–6 (30 s ITI). The observation that in this group SA rates were very close to control values on both the first two (2–6; 30 s ITI) and last three trials (7–9; 5 s ITI) suggests that, at this dose, β-CCM did not induce any change in the motivation to alternate. β-CCM produced a significant increase in SA rates only over Trials 2–3 to Trials 4–6 (Wilcoxon signed-rank: $P < .05$) despite an accumulation of proactive interference. This result suggests that β-CCM facilitates the expression of SA through a reduction in sensitivity to time dependent proactive interference. Finally, the memory enhancing effect of β-CCM at 0.5 mg/kg on SA rates was antagonised by RO 15-1788, suggesting thereby that it was mediated via BDZ receptors. This observation is reminiscent of studies showing that flumazenil antagonised the learning and memory effects of drugs acting on the BDZ receptors in both positively and negatively reinforced learning tasks (Stackman and Walsh, 1992). Interestingly, our present findings

are congruent with other studies involving spatial working memory in reinforced procedures and involving more effortful processing of information. However, the alternation procedure used in the present study involved a spontaneous processing of information, given the fact that there is no reinforcement and no training procedure. Thus, our finding showed that a memory task involving a spontaneous processing of information can be also affected by compounds acting on the GABA/BDZ receptors in animals.

4.2. Interaction between the emotional effects and the memory effects of the drugs

Our results suggest that, as a whole, SA rates tend to decrease with treatments that otherwise decrease anxiety levels. Indeed, the correlation performed for the entire population of the study (first experiment), between the emotional effects of the drugs and SA rates suggests that the emotional state of the subject is a possible factor underlying SA performance. More precisely, the reduction of anxiety in the open-field and the elevated plus-maze is associated with low SA rates on Trials 4 to 6, whereas the opposite pattern is observed with subjects exhibiting higher anxiogenic responses. It is interesting to observe that the positive correlation between anxiety scores and alternation performance are specifically observed on Trials 4–6, for which interference is greater as compared to the first trials (Trials 2–3) of the series. Interestingly, this positive correlation is also dependent on the delay interval separating trials (and not only on quantitative interference), since no correlation was observed between anxiety scores and alternation performance at Trials 7–9, separated by a 5-s interval. These distinctive features suggest an interaction between memory and levels of anxiety, since it depends closely on the place of the trials within the series and on the delay interval separating trials. Interestingly, the modulation of alternation performance is specifically linked to the intrinsic effects of the drugs, and is not dependent on differential influences of previous tests of anxiety since similar effects of the drugs on alternation scores are observed in the successive and independent testing procedures. This last finding is not congruent with reports showing large interactions between tests of anxiety and of spatial exploration delivered successively, but it has also been demonstrated that these between test interactions were highly strain dependent in the mouse (Rodgers and Cole, 1993). One can only conclude that such between test interaction is not observed in the Balb/c mouse strain, at least in the specific procedures we have used.

In conclusion, the present data show that both drugs (diazepam and β-CCM) bidirectionally modulate working memory in a task involving a low level of effortful processes and the emotional reactivity and that, in this task, the emergence of a positive correlation between anxiety and memory scores is dependent on certain time-dependent interference conditions (Trials 4–6).

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