Comparison between the effects of the benzodiazepine receptor ligands methyl beta-carboline-3-carboxylate and diazepam in two learning situations in mice

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Benzodiazepines are known to induce profound amnesia in man. In this report, we show, in mice, that whereas benzodiazepine diazepam indeed impairs performance, methyl beta-carboline-3-carboxylate (β -CCM), an inverse agonist of the benzodiazepine receptor, on the contrary enhances performance. The two learning situations used were one-trial passive avoidance and multiple-trial light-dark discrimination in a T-maze. Since these effects are blocked by Ro 15-1788, a specific antagonist of the benzodiazepine receptor, they are likely to be mediated by the benzodiazepine receptor. They seem to involve action on learning rather than on memory.

Benzodiazepines are widely used anxiolytics and anti-convulsants. Their potent sedative properties are routinely used in presurgical anesthesia. In the 1960s, anesthetists reported that benzodiazepines induced a strong anterograde amnesia in patients (Lister, 1985). Specific substances that can block the effects of benzodiazepines have recently been described (Braestrup, Nielsen, & Olsen, 1980; Hunkeler et al., 1981). Interestingly, aside from classical antagonists, some of these substances have intrinsic pharmacological properties that are opposite to those of benzodiazepines; they have been called inverse agonists (Braestrup, Schmiechen, Neef, Nielsen, & Petersen, 1982; Polc, Bonetti, Schaffner, & Haefely, 1982). Although benzodiazepines usually have an anticonvulsive activity, inverse agonists have been shown to be proconvulsant or convulsant (Oakley & Jones, 1982; Prado de Carvalho et al., 1984). Inverse agonists are also anxiogenic (Corda, Blaker, Mendelson, Guidotti, & Costa, 1983; Dorow, Horowski, Paschelke, Amin, & Braestrup, 1983; File, Lister, & Nutt, 1982; Ninan et al., 1982; Prado de Carvalho, Grecksch, Chapouthier, & Rossier,

1983), whereas benzodiazepines are anxiolytic. Finally, although benzodiazepines produce amnesia, several recent reports, including ours, show that inverse agonists enhance performance both in animals (Chapouthier, Venault, Prado de Carvalho, Simiand, & Rossier, 1984; Venault, Chapouthier, et al., 1986) and in humans (Duka, Stephens, Krause, & Dorow, 1987).

Our purpose in the present paper is to compare the effects of one of these inverse agonists, methyl beta-carboline-3-carboxylate (β -CCM) with those of benzodiazepine diazepam in mice. Two different situations are used: a one-trial situation (passive avoidance) and a multiple-trial situation (light-dark discrimination in a T-maze).

METHOD

Animals

The subjects were Swiss male mice (25–30 g) (CD1 Charles Rivers, France, for the passive avoidance task, and Iffa-Credo, France, for the choice situation). They were kept 10 per cage in our animal quarters at 25°C, on a 12:12-h light:dark cycle. All experiments were undertaken between 10:00 am and 4:00 pm.

Drugs

β -CCM, synthesized by one of us (R.H.D.), was dissolved in 0.1-N HCl and diluted to volume with saline. Diazepam, provided by Hoffmann-La Roche, Paris (1 mg/ml solution), was diluted to volume in saline. Ro 15-1788, kindly provided by W. Haefely (Hoffmann-La Roche, Basel, Switzerland), was suspended in sa-

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line with a drop of Tween 80. Drugs were administered subcutaneously (s.c.) or intraperitoneally (i.p.) in a volume of 0.05 ml/10 g (body weight).

Passive Avoidance Model

The mice were trained in a single-trial procedure. The animals were placed in an illuminated box connected to a dark box. Entrance into the dark box usually occurred within 30 sec and was punished by an electric footshock. On the next day (test session), the same mice were placed again in the illuminated box. Mice staying in the illuminated box for more than 60 sec were considered as remembering the task. Retention could thus be quantified by the percentage of animals avoiding the dark compartment.

The mice were injected with saline (s.c. or i.p.), β -CCM (0.1, 0.2, 0.3, and 0.4 mg/kg s.c.), or diazepam (1, 2, or 4 mg/kg i.p.). Either 15 min (saline, β -CCM) or 30 min (saline, diazepam) later, they were placed in an illuminated box (10 $\times$ 10 $\times$ 12 cm) connected to a large dark box (23 $\times$ 16 $\times$ 12 cm) with an electrified metallic grid floor. Upon stepping into the dark box, the mouse received a footshock. In order to obtain clear-cut effects, we decided to use two different situations: In experiments with β -CCM, where facilitation of retention was investigated, a low shock (0.1 mA for 2 sec, unscrambled dc current) was delivered. In experiments with diazepam where impairment of retention was investigated, a high shock (0.6 mA for 2 sec, unscrambled dc current) was delivered. In the β -CCM-treated groups, there were 20 subjects per group, except in the case of the control group (0.2 mg/kg), where this number was 40. The diazepam-treated groups and their control group each contained 12 subjects.

To investigate whether the effects of β -CCM and diazepam are mediated by the benzodiazepine receptor, an attempt was made to block them, in a second experiment, with the specific benzodiazepine antagonist Ro 15-1788 (Braestrup et al., 1980; Hunkeler et al., 1981).

Groups of mice ($n = 40$ for the low-shock groups; $n = 19$ for the high-shock groups) were injected before the first session with saline, β -CCM (0.3 mg/kg s.c.), diazepam (4 mg/kg i.p.), or Ro 15-1788 (15 mg/kg i.p.), or with a combination of β -CCM + Ro 15-1788 or of diazepam + Ro 15-1788. Ro 15-1788 was administered 15 min before the first session; the remaining injections were performed as described earlier.

Light-Dark Choice Situation

The training apparatus was a 4.5 $\times$ 4.5 cm section T-maze, consisting of a 15-cm-long departure alley and two 10-cm-long choice alleys. The walls and ceiling of the maze were made of transparent plastic. The floor consisted of a metallic grid that could be electrified. The three alleys could be electrified separately, and the connecting square was electrified together with the departure alley. The two choice alleys could be lit with a 25-W electric bulb situated above the ceiling.

Training consisted of six successive daily sessions of 10 trials each. Because our population of mice had a slight tendency to prefer the lit alley, the animals were trained to choose the dark alley. For a given trial, a mouse was placed at the entrance of the departure alley. It had 30 sec to choose between the lit or the dark alley. Past this delay, it was forced to choose by application of a 50- μ A 2-sec electric footshock. If it chose the lit alley, it received additional 50- μ A 2-sec electric footshocks separated by 2-sec intervals until it chose the dark alley. If it chose the dark alley, it could escape the maze, and it was, after 30 sec, placed at the entrance for another trial. An error was counted only when an animal entered the lit alley. Thus, only errors in discrimination were counted as errors. The speed of response was not investigated, since this discrimination procedure appeared to be a better measure. Light or darkness was distributed to the left or right arm of the maze according to a Gellerman's sequence.

In a first experiment, 10 min before the first three sessions, the animals were administered β -CCM (0.3 mg/kg, the most active dose in the passive avoidance model), diazepam (2.5 mg/kg), or saline. In a second experiment, again 10 min before each of the first three sessions, the animals received two simultaneous injections of β -CCM and Ro 15-1788, β -CCM and the Ro 15-1788 vehicle, Ro 15-1788 and the β -CCM vehicle, both vehicles, or one injection of saline. For the first experiment, the subjects numbered 20 in the control group and 16 in the other groups. In the second experiment, each group consisted of 16 subjects.

Statistical Analysis

The chi-square with Yates's correction allowed us to compare the percentages of the passive avoidance model. One-way analysis of variance was used in the light-dark choice situation.

RESULTS

Passive Avoidance Model

As is described in the Method section above, two different groups of mice were used. When facilitation of retention was to be investigated, a low-intensity shock was delivered; when impairment of retention was to be investigated, a high-intensity shock was used. In the low-shock group (Figure 1A), controls showed a poor level of retention,

Figure 1. Effects of β -CCM and diazepam on retention 24 h after a passive avoidance task in mice. The percentages of animals avoiding the dark box are presented in the figure. (A) $n = 20$ per group, except for controls and β -CCM 0.2 mg/kg, where $n = 40$. (B) $n = 12$ per group. * $p < .05$; ** $p < .01$, between treated and controls.

Table 1
Reversal of the effects of β -CCM by Ro 15-1788
in Passive Avoidance in Mice

Drug Injected	% Animals Avoiding
Saline	17.5
Ro 15-1788	20
β -CCM	40*
β -CCM + Ro 15-1788	15†

* $\chi^2 = 3.84$, $p < .05$, as compared with saline-treated. † $\chi^2 = 9.60$, $p < .001$, as compared with subjects treated with β -CCM alone.

and, during the test session, only a small percentage (12%) of subjects avoided the dark box. In this low-shock group, when β -CCM (0.2 and 0.3 mg/kg) was administered before the training session, a much higher percentage (35%) of the animals now avoided the dark box during the test session (as compared with controls, for β -CCM 0.2 mg/kg, $\chi^2 = 5.59$, $p < .05$; for β -CCM 0.3 mg/kg, $\chi^2 = 4.22$, $p < .05$). Lower (0.1-mg/kg) doses of β -CCM were less effective. The performance-enhancing effect of β -CCM in this task is in direct opposition to the amnesic effect of diazepam (1–4 mg/kg). Thus, in the high-shock group, the percentage of mice avoiding the dark box during the test session was reduced from 100% (controls) to nearly 40% (diazepam-treated) (Figure 1B) as compared with controls (for diazepam 2 mg/kg, $\chi^2 = 6.32$, $p < .05$; for diazepam 4 mg/kg, $\chi^2 = 9.88$, $p < .01$).

Control measurements showed that, during the training session, latency in entering the dark box did not differ

significantly under the influence of the different drugs ($M \pm SE$, in seconds): β -CCM controls, 9.8 ± 1.0 ; β -CCM 0.2 mg/kg, 8.8 ± 1.1 ; β -CCM 0.3 mg/kg, 10.8 ± 1.1 ; diazepam controls, 10.5 ± 0.9 , diazepam 2 mg/kg, 15.5 ± 2.6 ; diazepam 4 mg/kg, 16.3 ± 4.2 . Thus, differences in performance during the test session cannot be attributed to differences in latencies during the training session.

In a control experiment, shock was not delivered in the dark box. Instead, after having stepped into the dark box during the first session, the mice were returned to their home cages for 5 min; they were then shocked in a different lit box, thus dissociating the dark box from punishment. Twenty-four hours later, during the test session, no difference was observed between saline and treated subjects. The percentages of animals ($n = 15$ per group) avoiding the dark box were: saline, 8%; β -CCM 0.2 mg/kg, 0%; β -CCM 0.3 mg/kg, 17%. This control experiment rules out a nonspecific effect of β -CCM on avoidance of the dark box.

The results of the experiments with Ro 15-1788 (Table 1) showed that this compound had no effect when administered alone. However, the effects of β -CCM were completely antagonized by coadministration of Ro 15-1788. This could indicate that the effects of β -CCM are mediated by the benzodiazepine receptor.

Light-Dark Choice Situation

In the first experiment, the animals received saline, diazepam, or β -CCM. Analysis of variance provides evidence that all groups improved their performance in successive training sessions, as is shown by the difference in errors between the first three sessions and the last three [controls, $F(1,38) = 25.60$, $p < .001$; diazepam treated, $F(1,30) = 9.78$, $p < .01$; β -CCM treated, $F(1,30) = 14.75$, $p < .001$]. Global analysis of variance shows a significant effect of drugs for the first 3 days [$F(2,49) = 20.11$, $p < .001$], as well as for the last 3 without injection [$F(2,49) = 20.11$, $p < .001$]. Detailed analysis (Figure 2) reveals that performance is impaired by the administration of diazepam (increase in the number of errors), both during the sessions under drug treatment [diazepam vs. controls, $F(1,34) = 14.75$, $p < .001$] and during the sessions without drug [$F(1,34) = 10.50$, $p < .01$]. The opposite effect—that is, an improvement in performance (reduction of the number of errors)—was obtained with β -CCM, both during the sessions under drug treatment [β -CCM vs. controls, $F(1,34) = 7.13$, $p < .05$] and during the sessions without drug [$F(1,34) = 11.0$, $p < .01$].

In the second experiment, the animals received simultaneous administration of β -CCM + Ro 15-1788, β -CCM + vehicle of Ro 15-1788, Ro 15-1788 + vehicle of β -CCM, both vehicles, or saline. As before, all groups improved their performance with training, as is shown by the difference in errors between the first and last three sessions [controls, $F(1,30) = 56.96$, $p < .001$; vehicles, $F(1,30) = 34.08$, $p < .001$; β -CCM alone, $F(1,30) =$

Figure 2. Effects of diazepam and β -CCM on performance in maze learning. The bars represent the number of errors ($M \pm SE$) of the different groups of animals during the 30 trials of the first three sessions under drug treatment or of the last three sessions without drug. Open bars, controls ($n = 20$); hatched bars, diazepam-treated (2.5 mg/kg, $n = 16$); dotted bars, β -CCM-treated (0.3 mg/kg, $n = 16$). * $p < .005$; ** $p < .01$; *** $p < .001$, as compared with respective controls.

Table 2
Reversal of the Effects of β -CCM by Ro 15-1788
in the Light-Dark Choice Situation

Drug Injected	Errors During the 3 First Days (30 Trials) under Drug Treatment		Errors During the 3 Last Days (30 Trials) without Drug	
	<i>M</i>	<i>SE</i>	<i>M</i>	<i>SE</i>
Saline	13.5	0.6	6.8	0.6
Ro 15-1788	10.9	0.5*	4.25	0.7‡
β -CCM	10.2	0.7†	3.9	0.6§
β -CCM + Ro 15-1788	12.9	0.6	6.7	0.6
Both vehicles	13.3	0.6	7.4	0.9

Between treated and controls: * $F = 10.04$, $p < .01$. † $F = 13.54$, $p < .01$. ‡ $F = 7.75$, $p < .001$. § $F = 11.44$, $p < .001$.

53.33, $p < .001$; Ro 15-1788 alone, $F(1,30) = 62.42$, $p < .001$; β -CCM + Ro 15-1788, $F(1,30) = 54.95$, $p < .001$] (Table 2). Global analysis of variance shows a significant effect of the drugs during the first 3 days [$F(4,75) = 6.53$, $p < .001$], as well as during the last 3 days [$F(4,75) = 5.70$, $p < .001$]. Detailed analysis reveals that vehicle-treated groups did not differ from controls. Ro 15-1788 alone, as well as β -CCM alone, improved performance both during the sessions under drug treatment [β -CCM vs. controls $F(1,34) = 13.54$, $p < .001$; Ro 15-1788 vs. controls, $F(1,30) = 10.04$, $p < .001$] and during the sessions without drugs [β -CCM vs. controls, $F(1,34) = 11.44$, $p < .001$; Ro 15-1788 vs. controls, $F(1,30) = 7.75$, $p < .01$]. There was no difference, however, between the β -CCM + Ro 15-1788 group and the controls, during either the first three or the last three (without drug) sessions. Thus, Ro 15-1788 suppressed the effects of β -CCM administered alone.

DISCUSSION

Our results provide evidence for the enhancing effect of β -CCM on learning in both a one-trial passive avoidance task and a multiple-trial learning task. The action of diazepam was found to be directly opposite. If we consider that β -CCM acts at some stage of learning or memory processing, the question remains as to which stages of learning or memory are affected by the drug. If memory can be defined as the retention of information, this information needs to be acquired (learning) and then recalled (retrieval). In another behavioral task, habituation to a new environment (Venault, Chapouthier, et al., 1986), we showed that β -CCM had no effect when administered before the retention test. We could thus rule out an action on retrieval and suggest that β -CCM was acting on learning. This is in agreement with most results obtained with benzodiazepines in human subjects. But more direct proof was needed in animals. Using a light-dark choice situation, we verified that the effects of β -CCM observed using passive avoidance during the test session were already observable under drug treatment. As for passive avoidance, β -CCM improvement of performance was evident when the animals were no longer

under treatment (the last three sessions). But this improvement could already be seen during the administration of β -CCM (the first three sessions). This observation could not be made in the one-trial learning procedure. Though more work should still be done in this area, our data seem to rule out a state-dependency interpretation of the effects of β -CCM and diazepam. A state-dependency hypothesis would require that effects observed under drug treatment would not persist when the drug was no longer administered. Another objection could involve the electric footshock. It is well known that many treatments (e.g., ACTH, MSH, and even ECS) impair memory for training with intense footshocks and improve memory for training with weak footshocks. In the passive avoidance task, the fact that two levels of footshock were used could offer an alternative explanation for the difference observed between β -CCM and diazepam. Though this possibility cannot be excluded, it does not seem, however, to explain the observed effects completely. The fact that, for a given (fixed) footshock, the different doses of a drug produce different effects clearly suggests an action of the drug on learning that is independent of the possible action of the footshock. Furthermore, this objection cannot be proposed for the multiple-trial situation in which the footshock remains the same for the two drugs. On the other hand, it could also be possible that the two drugs have different analgesic properties that might contribute to the differences in learning. As far as β -CCM is concerned, however, our group has previously shown that a dose of 1-mg/kg β -CCM has no effect on pain threshold in mice (Prado de Carvalho et al., 1984). For β -CCM, we can thus rule out a possible explanation of this effect in terms of analgesia. But for diazepam, the question remains open. Finally, our present data confirm what we have previously suggested (Venault, Chapouthier, et al., 1986)—that is, that β -CCM has an action on acquisition rather than on retention (memory) itself.

Since, in the second experiment, the effects of β -CCM on learning were suppressed by administration of Ro 15-1788, which is a specific antagonist of the benzodiazepine receptor, it could be assumed that these effects are mediated by this receptor. A similar conclusion was drawn from the results obtained in the one-trial passive avoidance

task. Indeed, β -carbolines are known to bind with high affinity to the central benzodiazepine receptor (Braestrup et al., 1980; Guzman et al., 1984), and some have been proposed as endogenous ligands for these receptors (Braestrup et al., 1980; Pena, Medina, Novas, Paladini, & De Robertis, 1986). Ro 15-1788 has been described as a specific, high-affinity benzodiazepine receptor ligand that reaches its sites of action within the central nervous system very rapidly, preventing and reversing dose-dependently all effects that agonists and inverse agonists of the benzodiazepine receptor produce via this receptor (Haefely, 1988). It is, however, important to note that Ro 15-1788, classically used as a "neutral" antagonist of the benzodiazepine receptor, has effects of its own on learning behavior. Lal, Kumar, and Forster (1988) have shown an effect similar to that of β -CCM in another negatively reinforced learning task; these authors hypothesized that pretreatment with Ro 15-1788 "may facilitate learning or memory processes by reversing a negative modulatory influence of endogenous diazepam-like ligands for benzodiazepine receptors."

A classical interpretation of our data could be that in spite of their similar intrinsic actions, β -CCM and Ro 15-1788 antagonize each other when administered simultaneously; however, this would not necessarily mean that the effects of β -CCM and Ro 15-1788 are mediated by the same sites on the benzodiazepine receptor. An alternative interpretation could be proposed. It could be assumed that Ro 15-1788 enhances (instead of antagonizes) the effects of β -CCM. Thus, combined administrations of β -CCM and Ro 15-1788 could produce the same effect as higher doses of β -CCM alone—that is, a weaker effect on performance. Experiments with several doses of β -CCM and several doses of Ro 15-1788 could help to clarify this point. However, when doses of both compounds are high, it is known that Ro 15-1788 has a clear (classical) antagonistic effect (see File & Pellow, 1986, for review). Only synergy between very low doses of β -CCM and Ro 15-1788 could offer an answer to this point.

The question finally remains as to how β -CCM exerts its effects on learning. A first explanation of our data could be that β -CCM increases the level of arousal during the training session. This explanation would accord with the observation that, in rodents, arousal-enhancing drugs improve learning (Martinez, Jensen, & McGaugh, 1983) and that several β -carbolines increase arousal (Ongini, Barzaghi, & Marzanatti, 1983). The work by Jensen, Stephens, Sarter, and Petersen (1987) tends to confirm this view in rodents, by showing that cognitive effects of several BZ receptor ligands reflect changes in arousal or vigilance. Duka et al. (1987) found similar effects in man, where the β -carboline ZK 93 246 improved performances in two cognitive tasks: a "logical reasoning task and a picture differences task which estimated concentration and attention, respectively" (p. 421). This interpretation is, however, difficult in the case of chicks, where it has been found that, on the contrary, β -CCM has a sedative effect (Venault, Prado de Carvalho, et al., 1986). An explana-

Table 3
Effects of β -CCM and Diazepam on Performance During a Passive Avoidance Test 30 sec After Training

Drug Injected	% Animals Avoiding
Low-Shock Group	
Saline	10
β -CCM (0.3 mg/kg)	40*
High-Shock Group	
Saline	87
Diazepam (2 mg/kg)	27†

* $\chi^2 = 4.80$, $p < .05$. † $\chi^2 = 11.00$, $p < .001$ between treated and controls.

tion for this observation could be that the action of β -carbolines in birds is markedly different from that seen in mammals.

We tried to confirm this arousal-enhancing interpretation with an experiment in which we used the passive avoidance situation, the only difference being that the test session was performed 30 sec after the end of the training session instead of 24 h later. The high-shock groups consisted of 20 subjects, and the low-shock groups had 15 subjects. The data presented in Table 3 show that the results were the same as those obtained with a 24-h interval, suggesting an effect on learning.

The enhancing effects of β -CCM on learning could also be linked to its anxiogenic properties (and the amnesic effects of diazepam to its anxiolytic properties). However, in mice, where anxiogenic and convulsive effects of β -CCM as well as anxiolytic and anticonvulsive effects of diazepam have been extensively studied, the performance-enhancing effects of β -CCM were not seen in the dose range of the anxiogenic or convulsive effects of this drug. Indeed, the optimum dose for learning enhancement (0.2, 0.3 mg/kg) is much lower than the anxiogenic dose in a conflict model (1 mg/kg) or the convulsive doses (1–10 mg/kg) in the same strain of mice. It might nevertheless be assumed that the doses used in the present work, insufficient to produce noticeable effects with such classical techniques as conflict models, are still sufficient to act on an emotional component of performance. Similar reflections could be made for the action of diazepam. These explanations do not contradict an effect on learning and memory processes. They simply imply that the improvement of performance by β -CCM and the impairment of performance by diazepam in learning tasks on the one hand, and the effects in a conflict situation on the other, derive from common mechanisms involving anxiety.

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