

β -CCT, a selective BZ- ω_1 receptor antagonist, blocks the anti-anxiety but not the amnesic action of chlordiazepoxide in mice

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Received 24 November 1999; accepted as revised 12 January 2000

The aim of this study was to test further the hypothesis that different benzodiazepine (BZ- ω) receptor subtypes may mediate anxiolytic and amnesic effects of BZ agonists, using the selective BZ- ω_1 receptor antagonist β -CCT (β -carboline-3-carboxylate *t*-butyl-ester). Experiments were performed in Swiss mice using the elevated plus-maze anxiety test and two learning tasks – passive avoidance and the radial arm maze. In the elevated plus-maze test, β -CCT (30 mg/kg, i.p.) completely abolished the increase in open-arm entries induced by the BZ chlordiazepoxide (5 mg/kg, i.p.). Chlordiazepoxide decreased retention latency in the passive avoidance step-through procedure, and increased the number of errors in the radial arm maze. These effects were not modified by β -CCT. Except for a slight, albeit significant, amnesic effect in the passive avoidance test, β -CCT was devoid of intrinsic activity when administered alone. These results are in agreement with previous studies using selective BZ- ω_1 agonists, and thus provide further evidence that BZ- ω_1 receptors may be involved in the anxiolytic but not in the amnesic effects of BZ agonists. © 2000 Lippincott Williams & Wilkins.

Keywords: anxiety, memory, benzodiazepine, β -CCT, BZ- ω_1 receptors, elevated plus-maze, passive avoidance, radial arm maze, mouse

INTRODUCTION

Benzodiazepine (BZ) receptor agonists are widely used drugs in the clinical management of anxiety disorders. However, despite their therapeutic efficacy, BZs have come under critical review because of problems of drug dependence, tolerance, muscle relaxation and amnesia. In particular, the amnesic effects have been the focus of many investigations. It is now widely acknowledged that BZs induce anterograde amnesia in humans (Curran, 1991; Woods *et al.*, 1992; Barbée, 1993). In animal studies, BZs disrupt learning, as measured in passive or active avoidance tests (Thiébot, 1985; Venault *et al.*, 1986; Nabeshima *et al.*, 1990a), habituation procedures (Venault *et al.*, 1986; Nabeshima *et al.*, 1990b), discrimination models (Jensen *et al.*, 1987; Cole, 1990) and spatial learning procedures such as the Morris water maze (McNaughton and Morris, 1987; Anglade *et al.*, 1993) and the radial arm maze (Hodges and Green, 1986; Stackmann and Walsh, 1992;

Nakamura-Palacios and Roelke, 1997; Belzung and Dubreuil, 1998).

BZs produce their effects through an action at two distinct binding sites, both associated with the GABA_A receptor and called BZ₁ and BZ₂ receptors. These receptors have also been designated as ω_1 and ω_2 , respectively (Langer and Arbilla, 1988). Recent work has shown that there is considerable heterogeneity of GABA_A receptors. At least 15 different subunits have been identified in the mammalian CNS (α_{1-6} , β_{1-3} , γ_{1-3} , ρ_{1-2} and δ_1) and it is now widely acknowledged that the BZ- ω_1 subtype corresponds to GABA_A receptors containing the α_1 subunit, while the BZ- ω_2 subtype represents a heterogeneous population of receptors possessing α_2 , α_3 or α_5 subunits (reviewed by Luddens *et al.*, 1995; Sieghart, 1995). This difference at the molecular level has prompted speculation about a distinct involvement of these two categories of receptors in the behavioural response to BZs. Studies with selec-

tive BZ- ω_1 receptor agonists, such as zolpidem, zaleplon or abecarnil, have demonstrated that they have less propensity to produce ataxia and muscle relaxation than non-selective compounds. Moreover, there is evidence that chronic treatment with selective BZ- ω_1 receptor ligands produces little tolerance or gives rise to little physiological dependence after drug discontinuation. In addition, these compounds have been found to display anxiolytic-like effects in some animal models. Several studies also showed that, although BZ- ω_1 receptor agonists may produce cognitive effects, they usually occur at high and mostly sedative doses (reviewed by Sanger *et al.*, 1994; Griebel *et al.*, 2000). Evidence consistent with this idea has also been obtained with the selective BZ- ω_1 receptor antagonist β -CCT (β -carboline-3-carboxylate *t*-butyl-ester), which was found to block the anxiolytic, anticonvulsant, but not the myorelaxant, effects of BZs (Shannon *et al.*, 1984; Griebel *et al.*, 1999).

The present study aimed at investigating further the involvement of BZ- ω_1 and BZ- ω_2 receptors in the anti-anxiety and amnesic action of BZs. Experiment 1 was designed to confirm the ability of β -CCT to block the anxiolytic action of the BZ agonist, chlordiazepoxide (CDP). Experiment 2 assessed the ability of β -CCT to antagonize the amnesic effect of CDP, in an animal model designed to measure emotional memory, the passive avoidance test. Finally, Experiment 3 was performed to study the interaction between β -CCT and CDP using a radial arm maze, a memory test devoid of an emotional component. CDP was administered at 5 mg/kg and β -CCT at 30 mg/kg. These doses were chosen on the basis of previous work with the compounds (Belzung and Dubreuil, 1998; Griebel *et al.*, 1999). For example, the *in vivo* interaction of 30 mg/kg β -CCT with mouse brain BZ binding sites revealed that the drug prevented the binding of [³H]flumazenil in several brain regions. However, β -CCT interacted preferentially with BZ- ω_1 -enriched structures (i.e. cerebellum) than with BZ- ω_2 -enriched structures (i.e. spinal cord, hippocampus, striatum) at this dose.

METHODS

Subjects

Subjects were naïve male Swiss mice, aged 9 weeks at the time of testing. They were obtained from Janvier (Le Genest Saint Isle). All subjects were housed five per standard cage at a constant temperature of about 22°C under a reversed light/dark cycle (12/12 hours, lights off 08.00 hours). Commercial rodent pellets and tap water were freely available. The treatment of the animals was in accordance

with the European Community Council Directive 86/609/EEC.

Experiment 1: Interaction between chlordiazepoxide and β -CCT in the elevated plus-maze

The plus-mazes, made of yellowish polyvinylchloride, were elevated to a height of 38.5 cm and placed in a dark room. The open arms (27 × 5 cm) were lit by a 60 W transparent bulb, hanging 50 cm above each arm. Light intensity on the surface of the arms was about 550 lux. The closed arms (27 × 5 cm) had 15 cm high walls (also made of polyvinylchloride) and were covered with dark paper during the tests. All arms extended from a central platform measuring 5 × 5 cm. This experimental setup ensured a low proportion of entries onto the open arms, and has been shown to detect anxiolytic drug effects reliably, with a minimum confound of drug actions on ambulatory activity (Ågmo and Belzung, 1998; Belzung, 1999).

Tests began with the mouse being placed on the central platform with its head facing an open arm. Arm entries were registered on a hand-held computer (Psion Organiser) for 5 min. Later, the percentage of open-arm entries was calculated as the ratio open/total arm entries. The mouse was considered to be on an arm when all four paws were on the arm. Consequently, it was considered to be on the central platform whenever it had at least one paw on the platform. All tests were performed between the sixth and the ninth hour of the dark phase.

Experiment 2: Interaction between chlordiazepoxide and β -CCT in the step-through passive avoidance test

The apparatus consisted of a small rectangular LUCO runway (31.5 × 5 × 14 cm) that could be divided in two small compartments by a removable door. One compartment (7.5 cm long) was painted white, its top was transparent and it was well lit by a 100 W bulb placed above it. The other compartment (24 cm long) was painted black and darkened. The floor of both compartments consisted of steel bars spaced every 8 mm, that could be electrified. On the first day, mice were placed individually in the brightly illuminated white box, their heads facing the removable door. Thirty seconds later, the door was opened, allowing free access to the dark compartment. The latency to enter the dark side was recorded (learning latency). As soon as the mouse entered the dark compartment, the door was closed and, 15 s later, the animal was given a 0.2 mA foot shock delivered through the metal bars connected to a scrambler generator (Campden Instruments Ltd., shock source 521C) for 1 s. The mouse was removed from the dark

compartment 30 s after the end of the shock and placed in its home.

Twenty-four hours later each subject was again placed into the white box and the latency (retention latency) at entering the dark box previously associated with the foot shock was recorded up to 180 s. No shock and no treatment were delivered during this session.

Experiment 3: Interaction between chlordiazepoxide and β -CCT in the radial maze

The experimental device was an elevated maze with eight arms, 32 cm long and 5 cm wide, leading to an 8 cm square platform, radiating from a central circular platform 44 cm in diameter. A small cup, 1 cm in diameter, embedded in each distal platform, contained a hidden 10 mg noodle used as reinforcement. The apparatus was placed in a small room, on the walls of which four large black, white or black and white striped patterns hung, to provide particularly salient visual extramaze cues. (For further details on the apparatus, see Beuzen *et al.*, 1994.) Mice were progressively food-deprived so that weight loss reached 80–85% of their initial body weight at the beginning of testing.

Mice were first given two pretraining sessions at 24-hour intervals. Groups of four mice were placed on the maze at the same time and for 20 minutes per session, and could freely explore the eight arms, which contained abundant food.

Following pretraining, mice were given five training sessions, at 90-minute intervals. After baiting the eight arms with a 10 mg noodle, a mouse was placed on the central platform. The session ended when all eight arms had been explored or when 16 choices had been made. The total number of errors (when the mouse re-entered an already visited arm during the session) was recorded. After the passage of each mouse, the maze was quickly cleaned with Kleenex tissue to remove faecal deposits and urine.

Design

In all experiments, a parallel groups design was used, i.e. all doses of a drug and/or combinations of drugs included in a particular experiment were run in a single session. The order of drug treatments within a session was counterbalanced. There were 8–16 mice per group (see precise numbers in figure legends), and they were all experimentally naïve.

Drugs

Chlordiazepoxide HCl (purchased from Sigma, St. Louis, MO, USA) and β -CCT (synthesized by the chemistry department, Sanofi-Synthelabo) were dis-

solved in physiological saline. Saline was also injected into control animals. The drugs were injected intraperitoneally (i.p.) in a volume of 10 ml/kg. Each mouse received two injections 30 minutes before testing in the elevated plus-maze, the learning session in the passive avoidance test, or the fifth session in the radial arm maze.

Statistics

Because Bartlett's test for homogeneity of variance showed non-homogeneity in many cases, all data were analysed with the non-parametric Kruskal–Wallis ANOVA. *A posteriori* comparisons were made with the Mann–Whitney U-test.

RESULTS

Experiment 1: Interaction between chlordiazepoxide and β -CCT in the elevated plus-maze (Figure 1)

The Kruskal–Wallis test revealed a strong treatment effect on percentage of open-arm entries ($100 \times$ number of open-arm entries/total number of arm entries) and on number of closed-arm entries (respectively $H = 16.32$, $P = 0.001$; $H = 11.27$, $P = 0.01$). CDP increased both parameters. These effects were completely blocked by β -CCT. Mice treated with CDP and β -CCT differed from mice treated with CDP alone but not from controls. β -CCT did not elicit any intrinsic effect.

Experiment 2: Interaction between chlordiazepoxide and β -CCT in the step-through passive avoidance test (Figure 2)

The Kruskal–Wallis test showed that the treatments modified learning latency ($H = 8.65$, $P = 0.034$) as well as retention latency ($H = 10.32$, $P = 0.017$). *A posteriori* statistics showed that no groups differed for learning latency. CDP decreased retention latency, an effect that was not modified by β -CCT. β -CCT alone significantly reduced retention latency.

Experiment 3: Interaction between chlordiazepoxide and β -CCT in the radial arm maze (Figure 3)

The number of errors in the radial arm maze was significantly modified by treatments ($H = 11.70$, $P = 0.008$). This was due to the fact that CDP alone, and when combined with β -CCT, elicited an increase in the number of errors. β -CCT did not have any intrinsic activity.

DISCUSSION

The results of the present set of experiments confirmed that the BZ- ω_1 receptor antagonist β -CCT blocked some but not all behavioural actions of the

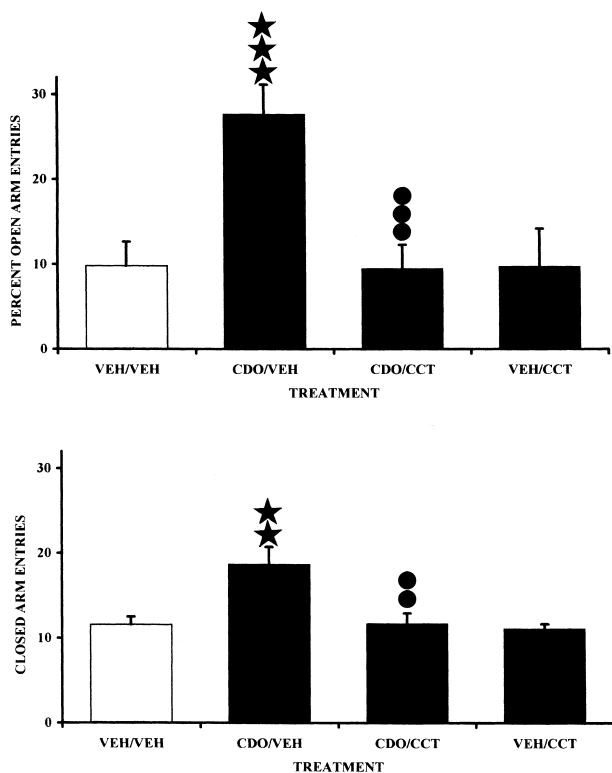


FIGURE 1. Interaction between CDP and β -CCT in the elevated plus-maze. Mean \pm SEM of percent of open-arm entries (upper panel) and number of closed-arm entries (lower panel), in mice, in the elevated plus-maze test. VEH/VEH: vehicle, $n = 16$; CDO/VEH: CDP, 5 mg/kg, $n = 14$; CDO/CCT: CDP (5 mg/kg) combined with β -CCT (30 mg/kg), $n = 15$; VEH/CCT: β -CCT (30 mg/kg), $n = 15$. **, ***: respectively, $P < 0.01$, $P < 0.001$: difference from controls. ••, •••: respectively, $P < 0.01$, $P < 0.001$: difference from mice treated with CDP alone.

non-selective BZ agonist, CDP. These findings further support the view that different BZ receptor subtypes may mediate different behavioural effects of BZs (Shannon *et al.*, 1984; Zivkovic *et al.*, 1992; Sanger *et al.*, 1994; Griebel *et al.*, 1999).

In the elevated plus-maze test, β -CCT, while displaying no effect by itself, blocked the anxiolytic and stimulant action of CDP. This finding is in agreement with those obtained in previous studies. For example, Griebel *et al.* (1999) showed that β -CCT abolished the anxiolytic effects of diazepam using the light/dark choice test. In another study, β -CCT was found to antagonize the anti-punishment action of diazepam (Shannon *et al.*, 1984). The present data therefore extend the previous results of other experimental procedures and another BZ (CDP instead of diazepam). It is to be noted that in our experiment, CDP increased both the percentage of open-arm entries, an index of anxiolysis, and the number of entries into the closed arm, which may be related to

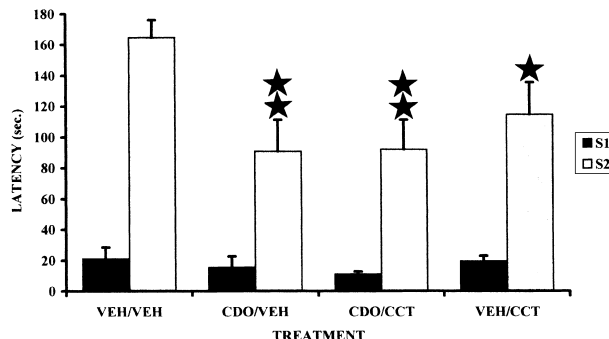


FIGURE 2. Interaction between CDP and β -CCT in the step-through passive avoidance test. Mean \pm SEM of latency to enter the dark compartment in the passive avoidance test. S1, Session 1; S2, Session 2 (S2 occurred 24 hours after S1). VEH/VEH: vehicle; CDO/VEH: CDP, 5 mg/kg; CDO/CCT: CDP (5 mg/kg) combined with β -CCT (30 mg/kg); VEH/CCT: β -CCT (30 mg/kg); $n = 15$ in all groups. Drugs were administered 30 min before S1. *, **: respectively, $P < 0.05$; $P < 0.01$: difference from controls.

an increase in locomotion. Such an association is not surprising because hyperactivity is probably a component of the expression of anxiolysis, as was shown by principal components analysis (Belzung and Le Pape, 1994).

In the passive avoidance test, β -CCT was unable to block the amnesic-like action of CDP. However, the effects of the BZ in this test could be attributed to an effect on the emotional component included in this procedure (the test involves fear of a place associated with a painful stimulus) rather than on memory *per se*. For that reason, the interaction

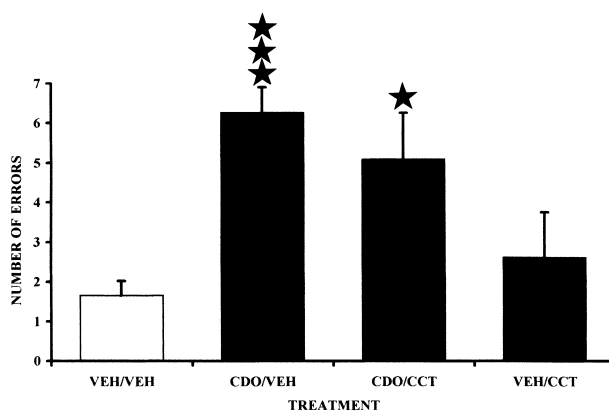


FIGURE 3. Interaction between CDP and β -CCT in the radial maze. Mean \pm SEM of number of errors during the fifth session by mice in the radial arm maze. VEH/VEH: vehicle, $n = 9$; CDO/VEH: CDP, 5 mg/kg, $n = 11$; CDO/CCT: CDP (5 mg/kg) combined with β -CCT (30 mg/kg), $n = 10$; VEH/CCT: β -CCT (30 mg/kg), $n = 8$. Drugs were administered 30 minutes before the fifth session. *, ***: respectively, $P < 0.05$; $P < 0.001$: difference from controls.

between CDP and β -CCT was also explored using the radial arm maze. The results showed that β -CCT also failed to block the amnesic action of the BZ in this latter model, thereby suggesting that the lack of action of β -CCT is not limited to a task involving emotional memory.

The interaction between β -CCT and CDP shown here contrasts with the results of previous studies that reported the interaction between BZs and the non-selective BZ antagonist, flumazenil. Indeed, flumazenil blocks the anxiolytic (Hunkeler *et al.*, 1981; Bonetti *et al.*, 1982; Belzung *et al.*, 1987) as well as the amnesic actions of BZs (De Noble *et al.*, 1990; McNamara and Skelton, 1992; Stackman and Walsh, 1992).

It is to be noticed that while β -CCT did not elicit any intrinsic activity in the elevated plus-maze and the radial arm maze, it induced an amnesic-like action in the passive avoidance test. A possible explanation for this dissociation may be linked to the fact that radial arm maze likely explores 'short-term memory' or 'working memory', while the passive avoidance step-through paradigm may be related to 'long-term' or 'reference memory'. Moreover, the first test evaluates emotional memory and the second test spatial memory. Further experiments including dose effects should permit confirmation of these data.

Previous studies with selective BZ- ω_1 receptor ligands have shown that these drugs may produce anxiolytic-like activities. This is best exemplified by the β -carboline abecarnil and the imidazopyridine zolpidem, which were found to produce anxiolytic-like effects in several models in mice and rats, including the four-plate test, the elevated plus-maze and/or the water-lick conflict test. However, both drugs were reported to be inactive in the free-exploration box, the light/dark test and in the defence test battery, three mouse models of anxiety, thereby questioning the contribution of BZ- ω_1 receptor in the anxiolytic activity of BZ agonists (Stephens *et al.*, 1990; Jones *et al.*, 1994; Stephens and Voet, 1994; Griebel *et al.*, 1996a, b, c, 1998). To unravel the issue, it was suggested that abecarnil and zolpidem have limited effects in anxiety tests because of their propensity to produce sedation, which may mask anxiolysis (Griebel *et al.*, 1996a, b, c).

In sharp contrast with the present and earlier pharmacological findings is a recent report by Rudolph *et al.* (1999), which suggests that BZ- ω_1 receptors are not primarily involved in the anxiolytic action of BZs, whereas this subtype may mediate the

amnesic effects of the drugs. These authors introduced a histidine to arginine point mutation of the murine α_1 subunit of the GABA receptor, which renders BZ- ω_1 receptors insensitive to BZs (α_1 mice). They showed that α_1 mice failed to exhibit the sedative, amnesic and partly the anticonvulsant action of diazepam, while the anxiolytic-like, motor impairing and ethanol-potentiating effects were fully retained. This discrepancy cannot be due to differences in behavioural devices, because these studies all used the same apparatuses (light/dark test and elevated plus-maze for anxiety, and passive avoidance for memory). It could, however, be due to differences in mice strains, because Rudolph *et al.* (1999) used 129/SvJ and C57BL/6J, while pharmacological studies used CD-1 and BALB/c (Griebel *et al.*, 1999) or Swiss (present experiments). Alternatively, it may be argued that the phenotype of α_1 mice may be the consequence of compensatory processes (Gerlai, 1996a, b, 1999), as was shown with other engineered animals such as 5-HT_{1B} knockout mice (Rocha *et al.*, 1998). Furthermore, it is clear that the effects of an acute antagonist cannot be equated with the chronic absence of a receptor which is found in knockouts (Crusio, 1996). For example, the 5-HT_{1B} receptor antagonist GR127935 was found to decrease the locomotor effects of cocaine in wild-type mice, while 5-HT_{1B} knockouts are more active in response to cocaine (Rocha *et al.*, 1998). Finally, one may consider the possibility that β -CCT acts via another target than BZ- ω_1 receptors. However, this is rather improbable, because binding studies revealed that β -CCT exhibits high binding affinity for α_1 -containing receptors, whereas it has low affinity for α_2 -, α_3 - or α_5 -containing receptors (Cox *et al.*, 1995).

In summary, the present findings provide some additional evidence for the functional significance of BZ receptor subtypes. They are consistent with the idea that α_1 -containing BZ receptors may be primarily involved in the anxiolytic action of BZ agonists, while α_2 -, α_3 - and/or α_5 -containing receptors may be associated with learning and memory, as was suggested in previous studies with selective BZ- ω_1 receptor agonists. Further experiments, including dose-response studies, may be necessary to confirm these data.

Acknowledgements

β -CCT was synthesized by Dr Yannick Evanno (Department of Medicinal Chemistry, Sanofi-Synthelabo). Experimental devices for the measurement of behaviour were built by Raymond Jegat (LEPCO).

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