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Bidirectional effects of benzodiazepine binding site ligands in the elevated plus-maze: differential antagonism by flumazenil and β-CCt

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Abstract

Recent research using genetically modified mice has pointed to the specific contribution of individual receptor subtypes to the various effects of benzodiazepines. The aim of this study was to examine the relative significance of α_1-containing $GABA_A$ receptors in the effects of modulators at the benzodiazepine site in the elevated plus-maze (EPM) under dim red light in rats. We tested the effects of the non-selective antagonist flumazenil (0–20.0 mg/kg), the preferential α_1-subunit selective antagonist β-carboline-3-carboxylate-*t*-butyl ester (β-CCt, 0–30.0 mg/kg), the non-selective agonist midazolam (0–2.0 mg/kg), the preferential α_1-subunit selective agonist zolpidem (0–2.0 mg/kg) and the non-selective inverse agonist methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM, 0–2.0 mg/kg). The influence of flumazenil (10.0 mg/kg) and β-CCt (30.0 mg/kg) on the effects of both kinds of agonists were also examined. The standard spatio-temporal parameters reflecting anxiety (percentage of open arm entries and time) and locomotion (closed and total arm entries) were analyzed.

β-CCt did not affect behavior, while flumazenil at the highest dose (20.0 mg/kg) decreased indices of open arm activity and total arm entries. Midazolam at the dose of 1.0 mg/kg significantly increased the percentage of open arm time, whereas at 2.0 mg/kg both anxiety-related parameters were increased. In contrast to the open arm entries, the open arm time was independent of the decreased closed arm entries, observed at 2.0 mg/kg. Flumazenil abolished these effects, whereas β-CCt partially potentiated the anxiolytic actions of midazolam. Zolpidem significantly increased both open-arm indices at 1.0 mg/kg, but the effect was dependent on the decreased closed arm entries. The selectivity of the anxiolytic-like effects of zolpidem was further checked under brighter white illumination. In these settings, the influence on anxiety-related, but not activity-related parameters, was absent. All of the activity-related effects of midazolam and zolpidem were mainly counteracted by both antagonists. DMCM produced significant anxiogenic effects at 1.0 mg/kg (open arm time) and 2.0 mg/kg (both parameters). β-CCt (30.0 mg/kg) and flumazenil at higher dose (20.0 mg/kg) antagonized the effects of DMCM.

The results indicate the anxiolytic effects of a non-selective benzodiazepine site agonist involve a predominant role of subunits other than α_1, whereas the behavioral indices of the anxiolytic-like properties of an α_1-selective ligand, if observed, depend on the experimental settings and the changes in locomotor activity, and hence were behaviorally non-specific. The present results generally correspond well to the behavioral findings with the genetically modified mice. On the other hand, the relative significance of the α_1-subunit in the anxiogenic effects of DMCM could not be clearly deduced.

Keywords: Anxiety; Benzodiazepines; $GABA_{A1}$ receptor; Elevated plus-maze

1. Introduction

Anxiety is a common emotional phenomenon in humans, which occurs in response to various stressors (Clement and Chapouthier, 1998). The response may

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include physiological (increase in heart rate, blood pressure, etc.), as well as behavioral (inhibition of ongoing behaviors, scanning, avoidance of the source of danger, etc.) parameters. Measuring anxiety-like behavior in animals is often based upon the exposure of subjects to unfamiliar aversive places (Belzung and Griebel, 2001). One major difficulty confronted in the experimental study of anxious disorders is the absence of concrete parameters reflecting "anxiety" per se. A common behavioral endpoint reflecting anxiety is a motor response (avoidance, escape, freezing and so forth), considered to express a mood state (Millan, 2003).

A remarkable diversity of mechanisms has been implicated in the etiology, modulation and treatment of anxiety. Prominent among them are GABAergic pathways, which exert an inhibitory influence upon the release and action of many neurotransmitters involved in anxiety generation (Millan, 2003). Importantly, not only neurons believed to participate in anxiety response are activated by the stress, but GABAergic neurons as well (Ishida et al., 2002; Millan, 2003). At $GABA_A$ receptors, the major receptor for this neurotransmitter, there are several modulatory sites, which mediate the actions of many drugs, among them benzodiazepines (Chebib and Johnston, 2000). Three kinds of allosteric modulators act through the benzodiazepine binding site: positive (agonist), neutral (antagonist) and negative (inverse agonist) modulators (Chebib and Johnston, 2000). Agonists and inverse agonists commonly exert bidirectional influences on behavioral parameters observed (Pellow and File, 1986; Stephens et al., 1987; Jensen et al., 1987; Chapouthier and Venault, 2002). In recent years, the complex structure and heterogeneity of $GABA_A$ receptors and benzodiazepine binding sites have been elucidated (Barnard et al., 1998). $GABA_A$ receptors are pentameric membrane proteins that operate as GABA-gated Cl^-channels, assembled from several families of subunits, of which at least 18 subunits occur in the CNS. The vast majority of receptors appear to be associations of two α-subunits, two β-subunits and a single γ-subunit, which comprise a central ion channel. The majority of them contain a benzodiazepine binding site located at the interface of the γ_2-subunit and the respective α-subunit (α_1, α_2, α_3 or α_5). These α-subunits contain a common feature: a conserved histidine residue in the drug-binding domain. Its conversion into an arginine residue renders the respective receptor diazepam-insensitive. Studies on mutant mice with converted residues pointed to the specific contribution of individual receptor subtypes to the pharmacological spectrum of benzodiazepines. Specifically, sedative and anterograde amnesic effects of benzodiazepines have been mainly attributed to α_1-containing $GABA_A$ receptor subtypes, anxiolytic action to the α_2-containing receptors, anticonvulsant activity, partially but not fully, to the α_1-containing receptors, and muscle relaxant effect largely to the α_2-containing receptors (Rudolph et al., 1999; McKernan et al., 2000; Low et al., 2000; Möhler et al., 2002; Rudolph and Möhler, 2004). A specific role of α_3-containing receptors in anxiolytic actions of benzodiazepines has also been advocated (McKernan et al., 2000; Reynolds et al., 2001; McKernan, 2002). In attempts to elucidate the functional relevance of structurally diverse $GABA_A$ receptor subtypes, the pharmacological approach, using subtype selective ligands, complements genetic studies, and is needed to corroborate and amplify insights provided by genetic studies (Millan, 2003).

Addressing these guidelines, we examined the effects of the non-selective agonist midazolam, the preferential α_1-subunit selective agonist zolpidem and the non-selective inverse agonist methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), on their own and in the presence of the non-selective antagonist flumazenil and the preferential α_1-subunit selective antagonist β-carboline-3-carboxylate-*t*-butyl ester (β-CCt), in a well-validated rodent model of anxiety, the elevated plus-maze (EPM) paradigm (Hogg, 1996). The EPM test is probably the most popular of all currently available animal models of anxiety, and affords an excellent example of a model based on the study of unconditioned, or spontaneous, behavior (Rodgers and Dalvi, 1997). Previous findings on the benzodiazepine site agonist–antagonist interactions in this model are inconclusive. In rats, flumazenil antagonized the anxiolytic-like effects of diazepam (Wada and Fukuda, 1991) and chlordiazepoxide (Ferris et al., 2001) applied systemically, and of midazolam microinjected into the dorsal periaqueductal grey matter (Russo et al., 1993). β-CCt was effective in antagonizing the facilitatory influences of chlordiazepoxide on open arm entries in mice (Belzung et al., 2000). On the other hand, flumazenil failed to antagonize the disinhibitory actions of diazepam on open arm behavior in mice (Dalvi and Rodgers, 1999) and even potentiated the anti-anxiety effects of midazolam applied into the dorsal raphé nucleus of rats (Gonzalez and File, 1997). In regard to flumazenil-β-carboline interactions in the EPM test, data are generally lacking.

The aim of the study was to systematically examine the capability of the differential influence of α_1-selective and non-selective antagonists on the, presumably (Pellow and File, 1986; Grahn et al., 1995), bidirectional effects of benzodiazepine site agonists and inverse agonists on spatio-temporal parameters in the EPM. As factor analysis demonstrates, the behavioral parameters in the rodent plus-maze provide measures of two independent factors, one reflecting anxiety and one reflecting motor activity. Percent open time and open entries load heavily on the factor taken to be anxiety, while the closed arm entries could be used as a relatively pure index of locomotor activity. Total entries load on the locomotor activity factor, but also, less heavily, on the anxiety factor (Lister, 1987; Cruz et al., 1994; Rodgers and Johnson, 1995; Fernandes and File, 1996; Ramos et al., 1997; Boguszewski and Zagrodzka,

2002; Yilmazer-Hanke et al., 2003). Analysis of the influence of the effects of substances on these parameters could serve as useful tools to assess the concomitant role of $GABA_{A1}$ receptor subtypes in mediating the effects on levels of anxiety and locomotor activity.

2. Materials and methods

2.1. Animals

Experiments were carried out on male Wistar rats (Military Farm, Belgrade, Serbia and Montenegro), weighing 200–240 g. All procedures in the study conformed to EEC Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Medical Faculty in Belgrade. The rats were housed in transparent plastic cages, six animals per cage, and had free access to pelleted food and tap water before and after drug administration. The temperature of the animal room was 22±1 °C, the relative humidity 40–70%, the illumination 120 lx and the 12/12-h light/dark period (light on at 06:00 h). All handling and testing took place during the light portion of the cycle. Throughout the study the animals were used only once.

2.2. Drugs

Midazolam and flumazenil were generously donated from F. Hoffman-La Roche (Basel, Switzerland). Zolpidem was purchased from Toronto Research Chemicals (North York, Canada) and DMCM from Research Biochemicals (Natick, MA, USA). β-CCt was synthesized as described in detail previously (Cox et al., 1995). All drugs were dissolved/suspended with the aid of sonication in a solvent containing 85% distilled water, 14% propylene glycol and 1% Tween 80, and were administered intraperitoneally, in a volume of 1 ml/kg, 20 min before testing. Doses are expressed as the base forms of the drugs. In the cases of combined treatment, agonists were administered at separate sites, immediately after the antagonist. Each animal received a total volume of 2 ml/kg of compounds tested or appropriate vehicles, at two different injection sites.

2.3. Behavior on the elevated plus-maze

The apparatus was constructed of sheet metal, with a black rubber floor. It consisted of a maze elevated to a height of 50 cm with two open (50×10 cm) and two enclosed arms (50×10×40 cm), connected by junction area (central platform) measured 10×10 cm. Although the floor was rubberized, a ledge of sheet metal (0.3 cm high) surrounding open arms was added, to avoid rats falling off. The illumination in the experimental room consisted of one red neon tube fixed on the ceiling, giving light intensity of 10 lx on the surface of the arms.

The experiments were carried out during the diurnal phase (between 08:00 and 12:00 h). At the beginning of the experiment, rats were placed in the centre of the maze, facing one of the enclosed arms and observed for 5 min. The observer sat in the same room 1 m from the maze. After each trial, the maze was cleaned with dry and wet towels. Throughout the study, the number of rats per treatment group was 8–14 (explicated in the legends of figures and in Table 1). Each experiment was run over 4 consecutive days, with three control rats per day; as there was no difference in plus-maze activity

Table 1
The effects (mean±S.E.M.) of zolpidem (Z: 0.5, 1.0 and 2.0 mg/kg) on the behavior of Wistar rats in the elevated plus-maze illuminated by 150 lx and the influence of flumazenil (F: 10 mg/kg) and β-CCt (β: 30.0 mg/kg) on these effects

Treatment	Total entries	Closed entries	% Open entries	% Open time
SOL	7.92±1.03	5.83±0.61	21.09±6.09	15.53±5.27
Z0.5	6.60±0.86	4.60±0.70	30.00±4.29	17.78±3.11
Z1.0	7.50±1.10	4.67±0.61	31.50±5.82	28.47±7.93
Z2.0	2.27±0.51*	1.55±0.47*	25.45±11.39	25.76±11.87
One-way ANOVA	$P<0.001$	$P<0.001$	$P=0.728$	$P=0.568$
Z0.5+F10	7.00 ±1.02	4.80±0.66	26.39±4.74	12.03±2.71
Z1.0+F10	7.20±1.02	5.10±0.67	25.56±3.69	11.23±3.03
Z2.0+F10	5.40±0.70	4.10±0.57	22.37±5.04	11.57±3.48
One-way ANOVA	$P=0.316$	$P=0.272$	$P=0.860$	$P=0.844$
Two-way ANOVA	$P=0.141$	$P=0.120$	–	–
Z0.5+β30	6.67±0.87	4.56±0.63	31.22±5.84	15.04±3.49
Z1.0+β30	6.00±0.89	4.20±0.66	34.09±4.53	14.57±3.99
Z2.0+β30	6.80±1.47+	4.90±0.72+	20.04±5.16	14.83±9.02
One-way ANOVA	$P=0.644$	$P=0.316$	$P=0.200$	$P=0.999$
Two-way ANOVA	$P=0.011$	$P=0.020$	–	–

* $P<0.05$ compared to solvent (SOL) group, Dunnet's test after one-way ANOVA (P-values given).

\+ $P<0.05$ compared to the corresponding effect of the agonist, Tukey's test after two-way ANOVA (P-values for agonist dose×antagonist interaction given). Ten rats were allocated to each treatment group, except for the solvent ($n=12$).

among control subgroups, they were pulled in a single control group (n=12).

In the first part of the study the effects of the solvent were assessed, in comparison with the saline control, as well as of antagonists: flumazenil (5.0, 10.0 and 20.0 mg/kg) and β-CCt (3.0, 10.0 and 30.0 mg/kg). Based on these experiments and data from the literature, doses of the antagonists were chosen for the following experiments.

In the second experiment, the effects of midazolam (0.5, 1.0 and 2.0 mg/kg) were evaluated, as well as the influence of flumazenil (10 mg/kg) and β-CCt (30 mg/kg) on the action of the agonist.

The third part of the study was designed to examine the effects of zolpidem (0.5, 1.0 and 2.0 mg/kg), and the influence of flumazenil (10 mg/kg) and β-CCt (30 mg/kg) on these effects. Additionally, in accordance with the obtained results, the experiment with zolpidem was replicated in the settings of brighter illumination (one white neon tube), giving light intensity of 150 lx on the surface of the arms.

In the fourth experiment, the inverse agonist DMCM (0.1, 0.5, 1.0 and 2.0 mg/kg) was evaluated, per se and also in the presence of flumazenil (10 mg/kg) and β-CCt (30 mg/kg).

The standard spatio-temporal variables, such as the number of entries into the open or enclosed arms, and the time spent on arms, were recorded. Arm entry and arm exit were defined as all four paws into and out of an arm, respectively. The behavioral parameters presented comprise the percentage of open arm entries [open entries/(open+closed entries)×100], the percentage of time spent in open arms [open arm time/(open+closed arm time)×100], the number of closed arm entries and the number of total arm entries.

2.4. Statistical analysis

All numerical data presented in the figures were given as the mean±S.E.M. Each dose–response curve (agonist or antagonist alone or agonist+antagonist) was assessed by a one-way ANOVA. If the ANOVA was significant, each treatment condition was compared with the appropriate solvent control by a Dunnett's test (α=0.05). In case of significant effect in the number of enclosed arm entries, an analysis of covariance (ANCOVA) was performed in the anxiety-related parameters using the number of enclosed arm entries as covariate. Interactions between the agonists and antagonists were analyzed separately with a two-way ANOVA [factors: agonist dose versus cotreatment (an antagonist or saline)]; pairwise comparisons for the assessment of the antagonist influence on the agonist effects were conducted by Tukey's test, one of the methods recommended even in the absence of an overall significant F-test (Wilcox, 1987). Statistical analyses were performed with commercial statistical software for PC, Stat for Windows R. 5.0.

3. Results

3.1. Experiment 1

The statistical analysis did not indicate any significant difference between saline and the solvent group. While β-CCt did not affect behavioral parameters measured, flumazenil significantly decreased the percentage of open arm entries [$F(3,43)=2.85$, $P<0.5$], the percentage of time on open arms [$F(3,43)=2.86$, $P<0.05$] and the number of total arm entries [$F(3,43)=5.64$, $P<0.01$]. In all cases, Dunnett's test for comparison to control (solvent group) revealed the significant effect of the highest dose tested (20 mg/kg) (Fig. 1).

3.2. Experiment 2

Midazolam significantly increased the percentage of open arm entries [$F(3,40)=2.85$, $P<0.05$]. Dunnett's test indicated that the effective dose of midazolam was 2.0 mg/kg. Interaction data were analyzed by a two-way ANOVA. For flumazenil as an antagonist, there were significant main effects of cotreatment [$F(1,53)=5.17$, $P<0.05$] and agonist dose by cotreatment interaction [$F(2,53)=3.28$, $P<0.05$]. Flumazenil fully antagonized the effect of midazolam at 2.0 mg/kg (Tukey's post-hoc test). Two-way ANOVA with β-CCt as a cotreatment did not reveal any influence of the antagonist (Fig. 2).

Midazolam also caused a significant increase in the percentage of time spent on open arms [$F(3,40)=4.86$, $P<0.05$]. The effective doses (Dunnett's test) were 1.0 and 2.0 mg/kg. For flumazenil as an antagonist, there were significant effects of cotreatment [$F(1,53)=8.82$, $P<0.01$] and agonist dose by cotreatment interaction [$F(2,53)=3.32$, $P<0.05$]. Flumazenil fully antagonized the effect of midazolam at 2 mg/kg (Tukey's test); the influence on the lower

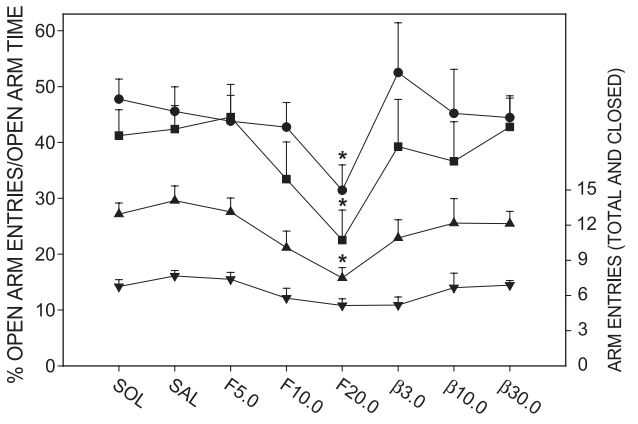

Fig. 1. The effects of flumazenil (F: 5.0, 10.0 and 20.0 mg/kg) and β-CCt (β: 3.0, 10.0 and 30.0 mg/kg) on the percentage of entries in the open arm (●), percentage of time spent in the open arm (■), total arm entries (▲) and closed arm entries (▼). *P<0.05 compared to solvent (SOL) group; SAL: saline group. Number of animals per treatment, for SOL through β30.0, respectively: 12, 12, 8, 13, 14, 8, 8 and 10.

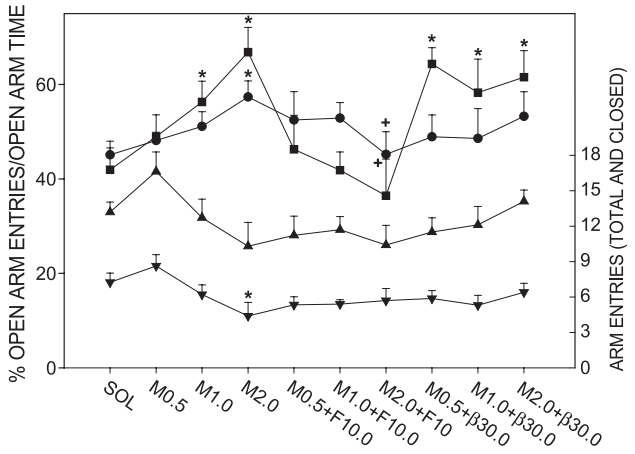

Fig. 2. The effects of midazolam (M: 0.5, 1.0 and 2.0 mg/kg) on the percentage of entries in the open arm (●), percentage of time spent in the open arm (■), total arm entries (▲) and closed arm entries (▼) (*P<0.05 compared to solvent (SOL) group) and the influence of flumazenil (F: 10 mg/kg) and β-CCt (β: 30.0 mg/kg) on these effects (^{+}P<0.05 compared to the corresponding effect of the agonist). Number of animals per treatment, for SOL through M2.0+β30.0, respectively: 12, 8, 14, 10, 9, 10, 8, 8, 9 and 10.

dose of midazolam tended to be significant (P=0.06). Two-way ANOVA with β-CCt did not reveal any influence of the antagonist. On the other hand, one-way ANOVA applied to the combination of midazolam+β-CCt showed a significant increase of the open arm time relative to control [F(3,35)=3.84, P<0.05]; the effect was significant (Dunnett's test) at all three levels of the agonist.

Midazolam influenced the number of closed arm entries [F(3,40)=3.15, P<0.05]. The parameter was significantly decreased at the dose of 2 mg/kg (Dunnett's test), and the complete antagonism of the effect was not observed (two-way ANOVA). However, midazolam×flumazenil and midazolam×β-CCt interactions tended to be significant [F(2,53)=2.90, P=0.06 and F(2,53)=2.95, P=0.06, respectively]. ANCOVA did not reveal a significant effect of midazolam to increase the percentage of open arm entries when the concomitant reduction in closed arm entries was taken into account [F(3,39)=1.25, P=0.30]. On the other hand, the increase of the percentage of time spent on open arms was independent of the decreased locomotor activity [F(3,39)=3.46, P=0.025].

3.3. Experiment 3

Zolpidem significantly increased the percentage of open arm entries [F(3,40)=2.84, P<0.05]. Dunnett's test indicated that the effective dose of zolpidem was 1.0 mg/kg. For flumazenil as an antagonist, there was a significant effect of zolpidem dose by cotreatment interaction [F(2,52)=3.24, P<0.05]; the effect of the antagonist as a factor tended to be significant [F(1,52)=3.29, P=0.08]. Tukey's test showed that the influence of flumazenil on the effect of zolpidem (1.0 mg/kg) was of borderline significance (P=0.06). In regard to β-CCt, there was a significant effect of cotreatment [F(1,52)=16.34, P<0.001], and the effective dose of zolpidem (1.0 mg/kg) was fully antagonized (Tukey's test) (Fig. 3).

Zolpidem also caused a significant increase in the percentage of time spent on open arms [F(3,40)=2.85, P<0.05]. For flumazenil as an antagonist, there was a significant effect of zolpidem dose by cotreatment interaction [F(2,52)=4.70, P<0.01]; Tukey's test showed that flumazenil antagonized the effect of zolpidem at 1.0 mg/kg (P<0.05). Two-way ANOVA showed a significant effect of zolpidem dose by β-CCt interaction [F(1,52)=3.43, P<0.05]; the effect of the antagonist as a factor tended to reach the significance [F(2,52)=2.99, P=0.09]. The influence on the effective doze of zolpidem was significant (P<0.05, Tukey's test).

Zolpidem influenced the number of total arm entries [F(3,40)=5.46, P<0.05]. Two-way ANOVA showed close to significant effects of flumazenil [F(1,52)=2.74, P=0.10] and zolpidem×flumazenil interaction [F(2,52)=3.07, P=0.06]. The effective dose of zolpidem (2.0 mg/kg, Dunnett's test) was fully antagonized by flumazenil (P<0.05, Tukey's test). In regard to β-CCt, two-way ANOVA showed a significant effect of zolpidem dose [F(2,52)=9.34, P<0.001] as well as of β-CCt as a factor [F(1,52)=5.67, P<0.05]; however, the influence on the effective dose of zolpidem was non-significant. Zolpidem significantly affected the number of closed arm entries [F(3,40)=5.80, P<0.05] as well. Dunnett's test indicated that the effective doses of zolpidem were 1.0 and 2.0 mg/kg. Two-way ANOVA showed the significant effects of flumazenil [F(1,52)=6.85, P<0.05] and zolpidem by flumazenil interaction [F(2,52)=4.220, P<0.05]. P values for post hoc comparisons were 0.04 (1.0 mg/kg zolpidem) and 0.10 (2.0 mg/kg zolpidem). In regard to β-CCt, two-way ANOVA revealed the significant effects of zolpidem dose

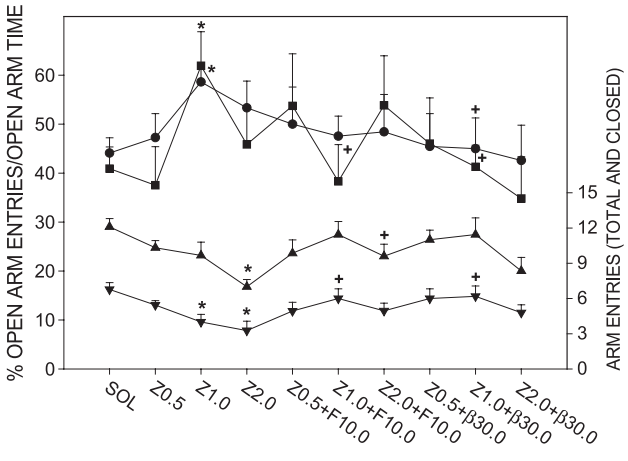

Fig. 3. The effects of zolpidem (Z: 0.5, 1.0 and 2.0 mg/kg) on the percentage of entries in the open arm (●), percentage of time spent in the open arm (■), total arm entries (▲) and closed arm entries (▼) (*P<0.05 compared to solvent (SOL) group) and the influence of flumazenil (F: 10 mg/kg) and β-CCt (β: 30.0 mg/kg) on these effects (^{+}P<0.05 compared to the corresponding effect of the agonist). Number of animals per treatment, for SOL through Z2.0+β30.0, respectively: 12, 8, 14, 10, 8, 8, 10, 8, 8 and 10.

[$F(2,52)=6.25$, $P<0.01$] and cotreatment [$F(1,52)=11.88$, $P=0.001$], but not of the interaction. Tukey's tests showed that β-CCt counteracted the effect of zolpidem at 1.0 mg/kg ($P<0.05$) but not at 2.0 mg/kg ($P=0.22$).

The significance of zolpidem effects on the percentage of open arm entries and the percentage of time spent in open arms disappeared when an ANCOVA was performed using the number of enclosed arm entries as covariate [respective F values: $F(3,39)=1.79$, $P=0.16$ and $F(3,39)=2.27$, $P=0.10$].

Since anxiolytic-like effects of zolpidem in red light appeared to be dependent on the decreased locomotor activity, an additional experiment under increased, white illumination was performed. The results are shown in Table 1.

In these settings, zolpidem (0.5–2.0 mg/kg) had no significant effects on the percentage of open arm entries [$F(3,38)=0.44$], or on the percentage of time spent on the open arms [$F(3,38)=0.68$]. Concomitantly, the drug significantly reduced the total number of arm entries [$F(3,38)=7.79$] and the number of closed arm entries [$F(3,38)=9.15$]. Post-hoc analyses showed that the depressant locomotor effects were induced by the highest tested dose of the agonist (2.0 mg/kg). Two-way ANOVA revealed that β-CCt (30 mg/kg) antagonized the influence of zolpidem on both activity-related parameters. Flumazenil (10 mg/kg) also tended to counteract the locomotor inhibition; the respective p values for post-hoc comparisons to the effective dose of zolpidem were 0.14 (total entries) and 0.05 (closed arm entries).

3.4. Experiment 4

DMCM significantly decreased the percentage of open arm entries [$F(4,49)=5.12$, $P<0.05$]. Dunnett's test indicated that the effective doses of DMCM were 1.0 and 2.0 mg/kg. For flumazenil as an antagonist, two-way ANOVA showed a significant effect of cotreatment [$F(1,70)=7.87$, $P<0.01$], but not of DMCM dose by flumazenil interaction. Post-hoc comparisons revealed no significant influence of the antagonist on the effective doses of DMCM (respective P-values 0.49 and 0.95). Two-way ANOVA showed a significant effect of β-CCt as a cotreatment [$F(1,83)=16.32$, $P<0.001$], but not of DMCM dose by β-CCt interaction. The influence of β-CCt on the effective doses of DMCM (1.0 and 2.0 mg/kg) partially reached the statistical significance (post-hoc P-values 0.13 and 0.05, respectively).

DMCM also caused a significant reduction in the percentage of time spent on open arms [$F(4,49)=3.60$, $P<0.05$]. The effective doses were 1.0 and 2.0 mg/kg (Dunnett's test). For flumazenil as an antagonist, two-way ANOVA showed a significant effect of cotreatment [$F(1,70)=6.66$, $P<0.05$], but not of DMCM by flumazenil interaction. Post-hoc comparisons revealed no significant influence of the antagonist on the effective doses of DMCM (respective P-values 0.11 and 0.85). Although there was again a significant main effect of β-CCt [$F(1,83)=10.11$, $P<0.01$], post-hoc comparisons failed to reach an acceptable level of statistical significance (P-values 0.11 and 0.09, against DMCM at 1.0 and 2.0 mg/kg, respectively).

One-way ANOVA revealed that, far different from DMCM on its own and in combination with flumazenil, the simultaneous administration of DMCM and β-CCt influenced the number of total arm entries [$F(4,56)=5.89$, $P<0.05$] and the number of closed arm entries [$F(4,56)=3.54$, $P<0.05$]. Dunnett's comparisons to control showed that the first parameter was decreased when two lower doses of DMCM (0.1 and 0.5 mg/kg) were combined with β-CCt (30 mg/kg), while closed arm entries were decreased when β-CCt combined with DMCM at 0.5 mg/kg. Two-way ANOVA for the combination of DMCM and β-CCt revealed a significant effect of the antagonist on the total arm entries [$F(1,83)=5.36$, $P<0.05$] and the closed arm entries [$F(1,83)=17.89$, $P<0.001$]. For the latter parameter, DMCM by β-CCt interaction was significant as well [$F(3,83)=5.48$, $P<0.05$]. Post-hoc comparisons revealed that the addition of β-CCt to DMCM dosed at 0.5 mg/kg significantly reduced both, the total and closed arm activity, compared to the inverse agonist per se (P-values 0.04 and 0.03, respectively).

The failure of flumazenil (10 mg/kg) to fully counteract the anxiogenic effects of DMCM prompted the experiment of concomitant administration of the inverse agonist and the non-selective antagonist at the anxiogenic doses on their own (2.0 and 20.0 mg/kg, respectively). For convenience, the results, corrected for the corresponding control values, were incorporated in Fig. 4. Student's unpaired t-test indicated the complete antagonism by the highest dose of flumazenil on the anxiogenic effects of DMCM.

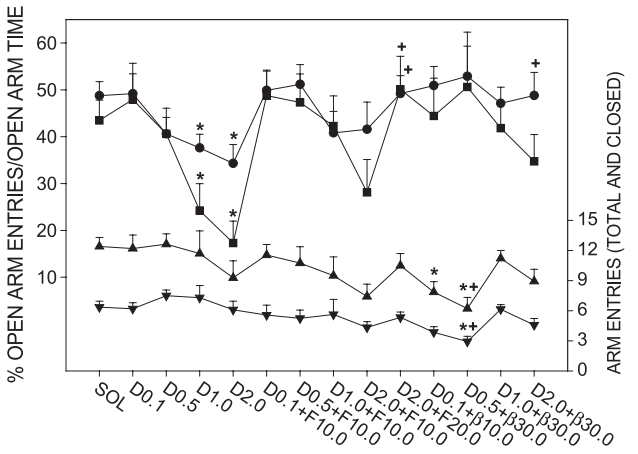

Fig. 4. The effects of DMCM (D: 0.5, 1.0 and 2.0 mg/kg) on the percentage of entries in the open arm (●), percentage of time spent in the open arm (■), total arm entries (▲) and closed arm entries (▼) (*$P<0.05$ compared to solvent (SOL) group) and the influence of flumazenil (F: 10 mg/kg) and β-CCt (β: 30.0 mg/kg) on these effects ($^{+}P<0.05$ compared to the corresponding effect of the inverse agonist). The interaction 2.0 mg/kg DMCM+20.0 mg/kg flumazenil added. Number of animals per treatment, for SOL through D2.0+β30.0, respectively: 12, 13, 8, 10, 11, 8, 8, 8, 12, 12, 8, 13, 14 and 14.

4. Discussion

The present experiments demonstrated that the non-selective (midazolam), but not the α_1-selective (zolpidem), benzodiazepine site agonist is able to exert anxiolytic effect in the rat EPM independent of the locomotor changes, whereas DMCM, a non-selective inverse agonist, elicited anxiogenic actions. The differential influence of the α_1-preferring (β-CCt) and the non-selective (flumazenil) antagonists on the bidirectional effects of benzodiazepine site ligands pointed further to the important role of subunits other than α_1 in mediating these effects on the emotional reactivity of the animal.

In regard to the experimental settings, the manipulation of the animals prior to testing and the aversiveness of the test conditions themselves are of enormous importance, especially in an effort to create baseline performance maximally sensitive to both anxiogenic and anxiolytic treatments (Grahn et al., 1995; Hogg, 1996). A major concern was avoidance of the flooring effect (behavioral parameters dependent on anxiety level so low that they cannot be significantly reduced further by anxiogenic drugs), often seen with inverse agonists (Hogg, 1996), noticed in our pilot examination under white light (150 lx), and confirmed in the experiment with zolpidem under brighter illumination. The reduction of light levels on the EPM has been reported repeatedly to decrease avoidance of the open arms (Griebel et al., 1993; Cardenas et al., 2001; Bertoglio and Carobrez, 2002a); however, this is not always observed (Becker and Grecksch, 1996; Jones and King, 2001). Obviously, bright light is a relative measure and it is likely that animals that are tested under light that is brighter than in their holding rooms will exhibit higher baseline anxiety (and, hence, increased sensitivity to anxiolytics), than those that are tested in low light, and vice versa (Hogg, 1996). Also, critically important could be the construction of the maze. The addition of ledges (0.3 cm high in our EPM) around the open arms influences the component of anxiety to which the apparatus is sensitive (Fernandes and File, 1996; Hogg, 1996). It affects the outcome of pharmacological manipulations, with a reduction in the anxiolytic effects of benzodiazepines (Jones and Cole, 1994; Fernandes and File, 1996) and an augmentation of the anxiogenic effects of inverse agonists (Jones and Cole, 1994). When compared with the similar experimental settings, especially regarding illumination and the rat strain, the control values from our experiments generally correspond well to the others (Cardenas et al., 2001; Bertoglio and Carobrez, 2002a,b,c).

In the first experiment, flumazenil at the highest dose (20 mg/kg) appeared to be anxiogenic. The profile of flumazenil in the rodent EPM is inconsistent: comparable doses of the antagonist are either inactive (Pellow and File, 1986; Moy et al., 1997) or anxiogenic (Lee and Rodgers, 1991; Da Cunha et al., 1992; Pokk and Zharkovsky, 1997). Inconsistency of its effects was observed in a number of other animal models of anxiety (reviewed in File and Pellow, 1986). The attempts to explain the effect lead to the hypothesis in regard to the influence on the actual conformational equilibrium during testing, with or without implicating putative endogenous ligands (File and Pellow, 1986; Malizia and Nutt, 1995). File and Hitchcott (1990) have proposed that the influence of flumazenil is dependent upon the anxiety state of the animal: under relatively low stress conditions, flumazenil would be predicted to exert either no- or anxiogenic effects, while in situations involving high levels of stress it would produce anxiolytic effects. As explained above, our test conditions should be considered as relatively low-anxiety.

The α_1-selective antagonist, β-CCt, in doses up to 30 mg/kg, did not affect the anxiety- nor locomotor activity-related parameters, in agreement with earlier studies in rats (Shannon et al., 1984) and mice (Griebel et al., 1999; Belzung et al., 2000). The interpretation of behavioral data is given an added dimension when considering the subtype-selective compounds: the degree to which the in vitro pharmacology of such ligands is reflected in vivo by differential, selective receptor occupancy should be discerned (Atack et al., 1999). β-CCt exhibits the greatest binding selectivity of the currently available $GABA_{A1}$-preferential ligands, with approximately 20-fold selectivity over the $GABA_{A2}$ and $GABA_{A3}$ receptors (Huang et al., 2000). Martin et al. (1989) showed that the total receptor binding per mg protein (B_{max}) of [^{3}H] diazepam added in vitro to the brain homogenates of rats treated with β-CCt at 30 mg/kg did not significantly decrease related to the vehicle- or 5 mg/kg β-CCt-dosed rats. Additionally, the binding reduction of radio-labeled flumazenil in mice previously dosed with 30 mg/kg of β-CCt followed the relative distribution of the α_1-subunit, which revealed retained selectivity of β-CCt binding (Griebel et al., 1999). Hence, for further experiments, the maximal behaviorally inactive doses of both antagonists were chosen.

The findings of anxiolytic-like actions of midazolam in the EPM generally replicated the results of earlier studies (Bertoglio and Carobrez, 2002b; Dal-Col et al., 2003). In our study, these effects were observed at somewhat higher doses than previously reported (Bertoglio and Carobrez, 2002b), presumably because of a shift in the sensitivity to the anxiolytic effects, related to addition of edging (Fernandes and File, 1996). In regard to locomotor activity, Bertoglio and Carobrez (2002b) observed decreased closed arm entries at the dose of 1 mg/kg, whereas in our study the effect was prominent at 2 mg/kg. Analysis of covariance (Pellow and File, 1986) showed that the influence of midazolam on the open arm time, but not on the open arm entries, was independent of the decreased locomotor activity.

Flumazenil mainly antagonized the anxiolytic actions of midazolam, whereas the reduction of closed arm entries was incompletely counteracted. Previous findings on the benzodiazepine site agonist-flumazenil interactions in the EPM

model are inconsistent. Flumazenil antagonized the anxiolytic-like effects of diazepam (Wada and Fukuda, 1991) and chlordiazepoxide (Ferris et al., 2001) applied systemically, and of midazolam microinjected into the dorsal periaqueductal grey matter (Russo et al., 1993) in rats. On the other hand, it failed to antagonize the disinhibitory actions of diazepam on open arm behavior in mice (Dalvi and Rodgers, 1999) and even potentiated the effects of midazolam applied into the dorsal raphé nucleus of rats (Gonzalez and File, 1997). In the social interaction test, flumazenil was able to antagonize the anxiolytic effects of midazolam administered into the dorsal hippocampus of rats (Gonzalez et al., 1998).

In contrast to the non-selective antagonist, β-CCt failed to block the effects of midazolam on the percentage of open arm entries; moreover, it potentiated the midazolam effects on the percentage of time spent on the open arms, revealing the anxiolytic effect of even the smallest tested dose of the agonist (0.5 mg/kg). In similar fashion to the influence of the non-selective antagonist flumazenil, β-CCt incompletely counteracted the depressant effect of midazolam on closed arm entries. The results are at odds with the finding that, in the EPM in mice, β-CCt, while displaying no effect by itself, blocked the anxiolytic and motor stimulant effects of chlordiazepoxide (Belzung et al., 2000). Similarly, β-CCt was shown to abolish the anxiolytic effects of diazepam in the light/dark choice test in mice (Griebel et al., 1999), and the anti-punishment action of diazepam in rats (Shannon et al., 1984). On the other hand, the present results are in accordance with findings from studies on mutant mice, in which the anxiolytic action of the benzodiazepines is mainly attributed to the α_2-containing $GABA_A$ receptor subtypes, while sedative (i.e. locomotor depressant) effects to the α_1-containing receptors (Rudolph et al., 1999; McKernan et al., 2000; Low et al., 2000; Möhler et al., 2002; Rudolph and Möhler, 2004). Moreover, Kralic et al. (2002) detected the anxiolytic effect of diazepam in the EPM paradigm at the lower dose in knockout mice devoid of the α_1-subunit (0.6 mg/kg) than in wild animals (1.0 mg/kg). Hence, it appears that the selective incapacitation of α_1-containing receptors could reinforce the anxiolytic action of the non-selective agonist, implying that the effects (possibly sedation) mediated by $GABA_{A1}$ receptors may actually interfere with the anxiolytic actions effected by non-α_1-containing receptors, in the paradigm employed here (EPM).

The question of possible anti-anxiety actions of the α_1-selective agonists is highly disputable. As CL 218,872, an α_1-preferring partial agonist, exerted anxiolytic-like effects in the punished drinking test in rats, Lippa et al. (1979) hypothesized that the α_1-subunit plays a crucial role in anti-anxiety effects of benzodiazepines. Accordingly, zolpidem appeared to be anxiolytic in the elevated plus-maze (Griebel et al., 1996a, 1998; Moy et al., 1997), in the Vogel's punished drinking test (Depoortere et al., 1986; Griebel et al., 1998), in hypertonic NaCl-solution drinking test (Lobarinas and Falk, 2000), in isolation-induced ultrasonic vocalizations, in rat pups (Olivier et al., 1998) and mouse pups (Rowlett et al., 2001). However, such effects were absent in the Geller-Seifter conflict procedure (Sanger and Zivkovic, 1986), in the test of food intake in novel environment (Perrault et al., 1990), in the light/dark test (Griebel et al., 1996b), in the mouse defense test battery (Griebel et al., 1996c) and in the open field test (Nazar et al., 1997). Lastly, zolpidem was devoid of activity against the social interaction deficits during ethanol withdrawal (Knapp et al., 2004).

In accordance with previous findings (Griebel et al., 1996a, 1998; Moy et al., 1997), zolpidem disinhibited open-arm behavior under dim red light at the dose of 1 mg/kg. The effect was mainly antagonized by both, the α_1-selective and the non-selective antagonist. In contrast to Griebel et al. (1996a), whose statistical analysis did not reveal any significant effect of zolpidem on closed/total arm entries, these parameters were reduced in our study. In a further study with the rat EPM, Griebel et al. (1998) concluded that zolpidem (and several other α_1-selective agonists) displayed anxiolytic-like activity at doses close to those producing behavioral suppression. In the EPM test in mice, the α_1-selective agonist alpidem was also shown to markedly reduce the total number of arm entries (Jones et al., 1994). As the open- and closed-arm changes were dependent in our study, an additional experiment with zolpidem was performed under brighter illumination, in an effort to create baseline performance more sensitive to the putative anxiolytic influences (Hogg, 1996). Despite the shift in behavioral baseline, zolpidem completely failed to exert the anxiolytic-like effects. In these settings, decreased locomotor activity was observed at the dose of 2.0 mg/kg, but not at 1.0 mg/kg. It could be argued that the more aversive situation of brighter illumination increased the level of arousal and (partly) counteracted the inhibitory effect on the motor activity. To be emphasized, the anxiolytic effects of the non-selective benzodiazepine site agonists have been repeatedly reported in the settings of moderate to bright illumination of the rodent EPM (e.g. Frussa-Filho et al., 1999; Belzung et al., 2000; Ferris et al., 2001). On the other hand, the anxiolytic-like effects of zolpidem and several other α_1-preferring agonists were obtained earlier under dim red light (Griebel et al., 1996a, 1998). The lack of these effects in the settings of brighter illumination, even at a dose devoid of locomotor interferences (1.0 mg/kg), implies that the capacity of zolpidem to counteract the enhancement of emotional reactivity is not only confounded by general activity changes (if present) (Griebel et al., 1996a), but is also inherently modest. The results with zolpidem fit well with the previously cited findings from studies on mutant mice (Rudolph et al., 1999; McKernan et al., 2000; Low et al., 2000; Kralic et al., 2002).

As the α_1-subunit is the major subtype, present in 60% of all $GABA_A$ receptors (Möhler et al., 2002), in virtually all brain regions (Pirker et al., 2000), its putative minor importance in anxiety control (Low et al., 2000; McKernan

et al., 2000) needs to be discussed. Although numerous interconnected limbic and cortical structures were implicated, the central importance in anxiety control was attributed to the amygdala (Millan, 2003). The distribution of α subunits in the amygdala is uneven; notably, the α_1-subunit is almost absent (Fritschy and Mohler, 1995; Kaufmann et al., 2003) or is modestly present (Pirker et al., 2000) in the central amygdala nuclei. Additionally, when co-assembled with the α_2-subunit (more than 35% of the total α_2-subunits in rat cortex and hippocampus), the α_1-subunit is pharmacologically inactive, i.e. such receptors do not possess the high-affinity binding site for zolpidem (del Rio et al., 2001). Accordingly, zolpidem potentiated GABA-evoked currents in the central amygdala nuclei only at high concentrations, far different from diazepam (Kang-Park et al., 2004).

The inability of antagonists to fully counteract the reduction of parameters dependent on locomotor activity, observed to similar degree with both midazolam and zolpidem under red light, is not unequivocally explainable. However, the influences of antagonists were often of borderline significance, while β-CCt (but not flumazenil) completely reversed the activity-related effects of zolpidem in the experiment under elevated light level. These findings could reflect subtle shifts in the patterns in which behavioral parameters depend on different dimensions (i.e. factors) of behavior, based on the test conditions and the kind of treatment used.

In agreement with previous findings in the rodent EPM paradigm (Rago et al., 1988; Cole et al., 1995; Grahn et al., 1995), DMCM exerted an anxiogenic-like activity. The effective doses (1.0 and 2.0 mg/kg) corresponded well with those found by Cole et al. (1995): 1.25 and 2.5 mg/kg. However, in contrast to the significant reduction in total arm entries reported by Cole et al. (1995), this parameter, despite a trend, was not significantly affected in our study. The lack of changes in closed arm entries replicated the findings with the inverse agonist FG 7142 and indicated the behavioral specificity of the anxiogenic effect (Rodgers et al., 1995).

At the dose of 10 mg/kg, flumazenil only partially antagonized the anxiogenic actions of DMCM. This finding prompted the extension of the experiment using flumazenil at the anxiogenic dose of 20 mg/kg itself. At this dose, flumazenil completely antagonized the action of DMCM. As far as we know, there are no data in regard to flumazenil-β-carbolines interactions in the EPM test. In other paradigms, flumazenil mainly antagonized the anxiogenic effects of various β-carbolines (Barrett et al., 1985; Belzung et al., 1987; De Boer et al., 1992). In the open field test, a relatively low dose of flumazenil (3.6 mg/kg) failed to antagonize the freezing effect of β-CCB in rats (Novas et al., 1988). In agreement with our results, flumazenil was able to antagonize the anxiogenic effects of the partial inverse agonists FG 7142 and β-CCE in the social interaction test even at doses at which it was itself anxiogenic (File and Lister, 1983; File and Pellow, 1984).

β-CCt tended to counteract the action of DMCM on anxiety-related parameters; in addition, the selective blockade of α_1-containing receptors resulted in reduction of activity-related parameters, especially salient when combined with 0.5 mg/kg DMCM. The differences in the overall behavioral interaction between DMCM and β-CCt, related to flumazenil combined at 10 and 20 mg/kg, could be explained in two ways. Firstly, DMCM-elicited anxiogenic effects might be dominantly mediated through the α_1-subunit, and hence to a great degree susceptible to counteraction by β-CCt. However, the motor interaction could not be explained in that case. As a more probable alternative, β-CCt would additionally act through mechanism(s) unrelated to flumazenil, but important, when interacting with DMCM, for the actions on anxiety- as well as activity-related parameters. In that case, DMCM-elicited anxiogenic effects would be dominantly mediated through the non-α_1, but (still) flumazenil-sensitive subunits, such as α_2 or α_3, so being liable to the dose-dependent blockade by flumazenil.

DMCM can interact with a distinct $GABA_A$ receptor via at least two separate sites: a high affinity site responsible for negative modulation, and a low affinity site, flumazenil-insensitive, which mediates positive modulation (Sigel et al., 1990; Im et al., 1995; Stevenson et al., 1995). The strong positive GABA modulating effects observed with β-CCt at concentrations >10 μM (Lüddens, June, Cook, unpublished; cited in June et al., 2003) could be correlated with previous findings. The significance of these in vitro results for behavioral studies is under study and would require sufficiently high levels of β-carbolines bound to the low affinity site.

Finally, when analyzing multiple subtypes of $GABA_A$ receptors, in the sense of affinities and efficacies of binding sites for benzodiazepines, flumazenil, β-CCt and DMCM (Barnard et al., 1998; Huang et al., 2000; June et al., 2003), it should be noted that the γ_1 subunit introduces atypical modulatory effects (Barnard et al., 1998). Namely, GABA action at γ_1-containing receptors is potentiated by DMCM (Puia et al., 1991; Wafford et al., 1993), more efficaciously at $\alpha_1\beta_1\gamma_1$ than at $\alpha_2\beta_1\gamma_1$ receptors (Puia et al., 1991), but is insensitive to flumazenil (Wafford et al., 1993). Pirker et al. (2000) reported on the high concentrations of γ_1-subunit-immunoreactivity in the central and medial nuclei of amygdala in rats. It is tempting to speculate that γ_1-containing receptors, which possess distinct pharmacological properties, could be important in mediating a part of anxiety-related actions of DMCM. The lack of data about β-CCt binding and efficacy at γ_1-containing receptors precludes, as yet, further discussion about the possible neurobiological consequences of γ_1-mediated actions, and further studies are required.

In conclusion, the present results indicate that for the anxiolytic effect of a non-selective benzodiazepine site agonist predominant role have subunits other than α_1, whereas the behavioral indices of the anxiolytic-like

influence of an α_1-selective ligand, if observed, depend on the experimental settings and the changes in locomotor activity, and hence were behaviorally non-specific. The present results generally correspond well to the behavioral findings with the genetically modified mice. On the other hand, the relative significance of the α_1-subunit in the anxiogenic effects of DMCM could not be clearly deduced.

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