Research report

βCCT, an antagonist selective for α_1GABA$_A$ receptors, reverses diazepam withdrawal-induced anxiety in rats

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ABSTRACT

The abrupt discontinuation of prolonged benzodiazepine treatment elicits a withdrawal syndrome with increased anxiety as a major symptom. The neural mechanisms underlying benzodiazepine physical dependence are still insufficiently understood. Flumazenil, the non-selective antagonist of the benzodiazepine binding site of GABA$_A$ receptors was capable of preventing and reversing the increased anxiety during benzodiazepine withdrawal in animals and humans in some, but not all studies. On the other hand, a number of data suggest that GABA$_A$ receptors containing α_1 subunits are critically involved in processes developing during prolonged use of benzodiazepines, such are tolerance to sedative effects, liability to physical dependence and addiction. Hence, we investigated in the elevated plus maze the level of anxiety 24 h following 21 days of diazepam treatment and the influence of flumazenil or a preferential α_1-subunit selective antagonist βCCt on diazepam withdrawal syndrome in rats. Abrupt cessation of protracted once-daily intraperitoneal administration of 2 mg/kg diazepam induced a withdrawal syndrome, measured by increased anxiety-like behavior in the elevated plus maze 24 h after treatment cessation. Acute challenge with either flumazenil (10 mg/kg) or βCCt (1.25, 5 and 20 mg/kg) alleviated the diazepam withdrawal-induced anxiety. Moreover, both antagonists induced an anxiolytic-like response close, though not identical, to that seen with acute administration of diazepam. These findings imply that the mechanism by which antagonism at GABA$_A$ receptors may reverse the withdrawal-induced anxiety involves the α_1 subunit and prompt further studies aimed at linking the changes in behavior with possible adaptive changes in subunit expression and function of GABA$_A$ receptors.

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1. Introduction

Benzodiazepines are reasonably safe and effective drugs in short-term treatment of different psychiatric and neurological disorders (anxiety, sleep disturbances, muscle spasms and seizure disorders). However, their protracted use remains debatable because it is associated with development of tolerance to some of their effects, liability for physical dependence and abuse potential. Abrupt cessation of prolonged benzodiazepine treatment is followed by a withdrawal syndrome, the main indicator of physical dependence. Benzodiazepine withdrawal has been characterized by many signs (e.g., anxiety, insomnia and in more severe cases seizures) that are opposite to their expected therapeutic effects. Despite the half-century long clinical and experimental experience, the molecular and neurobiological mechanisms underlying the development of benzodiazepine dependence are still insufficiently understood (Dell'osso and Lader, 2012; Licata and Rowlett, 2008).

Non-selective benzodiazepines, such as diazepam, bind to GABA$_A$ receptors containing α_1, α_2, α_3 or α_5 subunits in addition to the γ_2 subunit and allosterically modulate their activity. Therefore, it could be expected that alterations in GABA$_A$ receptor function provide possible mechanisms for the development of tolerance and physical dependence (reviewed in Bateson, 2002). A number of in vitro and ex vivo studies have suggested that abrupt withdrawal from the prolonged exposure to benzodiazepines results in expression changes of distinct GABA$_A$ receptor subunits (reviewed in Uusi-Oukari and Korpi, 2010). These changes could be associated with a decrease of postsynaptic GABA sensitivity (Gallager et al., 1984), or "functional uncoupling" between the recognition sites of benzodiazepines and GABA (Ali and Olsen, 2001; Hu and Ticku, 1994). More recently, it has been proposed that GABA$_A$ receptors containing α_1 subunits (α_1GABA$_A$ receptors) are critically involved in processes that underlie tolerance to the sedative effect, liability to physical dependence and addiction (Mirza and Nielsen, 2006; Tan et al., 2010; van Rijnsoever et al., 2004). However, the

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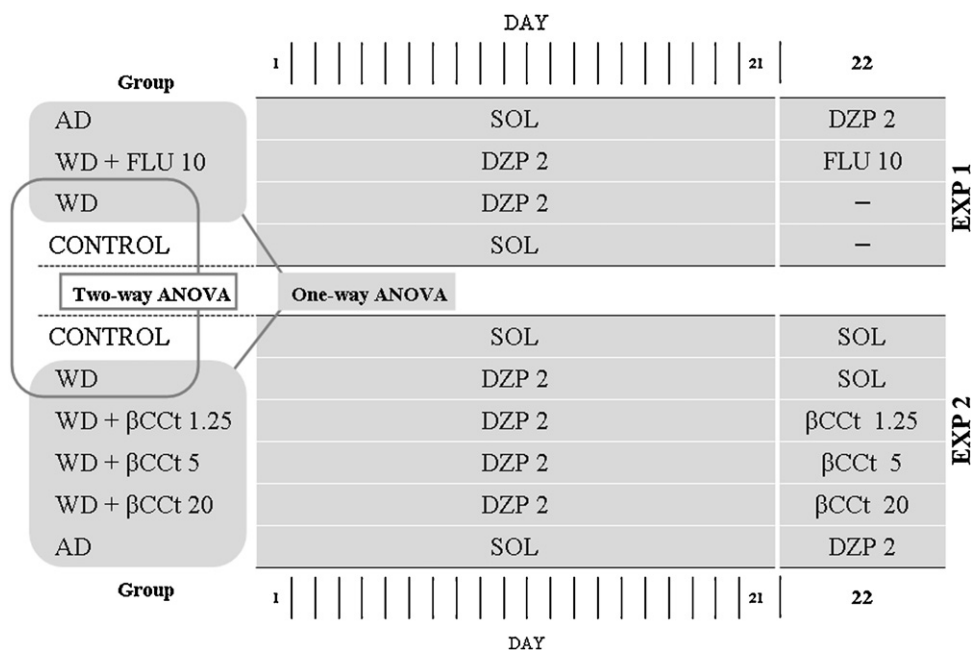E-mail address: miroslav@pharmacy.bg.ac.rs (M.M. Savić).

Fig. 1. The schematic presentation of experimental design and statistical analysis. AD, acute administration of diazepam; WD, diazepam withdrawal; FLU, flumazenil; SOL, solvent; DZP, diazepam; EXP, experiment.

phenomena such as withdrawal and dependence liability are hardly explainable on the basis of the neuronal adaptations at the level of GABA$_A$ receptors only, and involvement of other neurotransmitters, such as glutamate and dopamine, has been also implicated (Allison and Pratt, 2003; Diaz et al., 2011).

Flumazenil is a non-selective antagonist of the benzodiazepine binding site of GABA$_A$ receptors. In previous studies, the administration of flumazenil after prolonged benzodiazepine treatment tended to exert an ambiguous influence on the anxiety level. Namely, while some experiments showed that flumazenil can precipitate withdrawal symptoms, there was also evidence that flumazenil is capable of preventing and reversing the increased anxiety during benzodiazepine withdrawal in animals and humans (File and Hitchcott, 1990; Hood et al., 2009; Licata and Rowlett, 2008). File and Hitchcott (1990) have proposed that the anxiety level of the subject determines the direction of the flumazenil's influence on anxiety: when this is high, such as during benzodiazepine withdrawal, flumazenil would become anxiolytic; when this is low, flumazenil would increase anxiety. However, the receptor mechanism by which flumazenil affects anxiety during benzodiazepine withdrawal is still not established.

In laboratory animals, physical dependence to benzodiazepines may be observed as the emergence of characteristic symptoms, such as withdrawal anxiety, 12, 24 or 48 h upon abrupt cessation of the prolonged treatment (dos Santos et al., 2010; Licata and Rowlett, 2008). An anxiogenic response to benzodiazepine withdrawal in rodents is most commonly assessed using the elevated plus maze (EPM), and quantified by a decrease in the percentage of open arm entries and the percentage of time spent in open arms (dos Santos et al., 2010; File et al., 1987). Based on the proposed role of α_1-containing GABA$_A$ receptors in alterations in GABA-ergic neurotransmission following prolonged exposure to benzodiazepines, and also the observed bidirectional effects of flumazenil, it is worth to investigate in parallel the influence of a non-selective antagonist (flumazenil) and a preferential α_1-subunit selective antagonist (βCCt) on diazepam withdrawal syndrome. In the present study, we assessed in the EPM the level of anxiety 24 h following 21 days of diazepam treatment and examined the influence of flumazenil and βCCt on rat behavior during diazepam withdrawal. The doses

of antagonists were chosen in accordance with our previous studies, which demonstrated that flumazenil applied in doses up to 20 mg/kg and βCCt applied in doses up to 30 mg/kg were behaviorally inactive on their own in the EPM test (Savić et al., 2004).

2. Materials and methods

2.1. Animals

Experiments were carried out on 74 male Wistar rats (Military Farm, Belgrade, Serbia), weighing 180–200 g at the beginning of experiments. All procedures in the study conformed to EEC Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Faculty of Pharmacy in Belgrade. The rats were group-housed (six per cage) in transparent plastic cages with tap water and food pellets available *ad libitum* and kept in standard laboratory conditions. The temperature of the animal room was $22 \pm 1\,°C$, the relative humidity 40–70%, the illumination 120 lx, and 12/12 h light/dark period (light on at 6:00 h). All handling, daily administration of treatment and testing took place during the light period of the cycle, between 9:00 and 13:00 h.

2.2. Drugs

The compounds used in this study were diazepam (Galenika, Serbia), the non-selective GABA$_A$ antagonist flumazenil (Feicheng BoYuan Fine Chemicals Co., Ltd, China) and the preferential α_1-subunit affinity selective GABA$_A$ antagonist βCCt (t-butyl-β-carboline-3-carboxylate), synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, USA. The compounds were dissolved/suspended in a solvent containing 85% distilled water, 14% propylene glycol, and 1% Tween-80 and were administered intraperitoneally (IP) in a volume of 1 ml/kg.

2.3. Experimental procedure

Two separate experiments were performed, as illustrated in Fig. 1. In Experiment 1, the rats were randomly divided into four pre-treatment groups: two groups were repeatedly treated once daily with diazepam (2 mg/kg/day) and the other two groups received solvent IP, during 21 days. On the testing day (22nd day), 24 h after the last pre-treatment injection, the animals repeatedly treated with diazepam were acutely challenged with flumazenil 10 mg/kg or did not receive any treatment. The rats repeatedly treated with solvent during 21 days, received 2 mg/kg diazepam or did not receive any treatment on the testing day.

In Experiment 2, another set of experimentally naïve animals was randomly distributed among six pre-treatment groups: four groups were repeatedly treated once daily with diazepam (2 mg/kg/day), and the other two groups received treatment with solvent during 21 days. On the testing day, 24 h after the last pre-treatment injection, animals repeatedly treated with diazepam were acutely challenged with either solvent or βCCt at a dose of 1.25 mg/kg, 5 mg/kg or 20 mg/kg. On the other

Table 1
The effects of diazepam withdrawal on behavior in the EPM and the accompanying two-way ANOVAs with treatment groups and experimental conditions as factors. Treatment groups were control (SOL) and diazepam-withdrawn (WD) groups, whereas experimental conditions were Experiment 1 and Experiment 2. Data are mean ± SEM; number of animals per treatment group was 6–8.

| Parameter | Experiment 1 | | Experiment 2 | | Two-way ANOVA | | | | | |
| | SOL | WD | SOL | WD | Treatment group (SOL vs. WD) | | Experimental condition (Experiment 1 vs. Experiment 2) | | Interaction | |
					$F_{(1,25)}$	P	$F_{(1,25)}$	P	$F_{(1,25)}$	P
% open time	43.50 ± 3.34	28.86 ± 6.48	46.67 ± 8.16	31.25 ± 6.70	5.85	0.023	0.20	0.659	0.01	0.951
% open entries	41.63 ± 2.58	33.71 ± 4.47	37.00 ± 5.20	34.25 ± 3.56	1.86	0.085	0.27	0.606	0.44	0.516
Time in distal part of open arms (s)	36.49 ± 3.24	20.67 ± 8.00	38.43 ± 8.75	24.60 ± 8.26	4.18	0.052	0.16	0.689	0.02	0.899
Risk assessment behavior (s)	33.50 ± 6.70	49.91 ± 7.43	39.78 ± 13.70	62.05 ± 12.61	3.45	0.075	0.78	0.385	0.08	0.781
Total distance traveled (m)	10.31 ± 0.86	9.56 ± 0.51	9.65 ± 0.65	9.26 ± 0.52	0.71	0.407	0.51	0.480	0.07	0.781
Total entries	12.88 ± 0.95	11.43 ± 0.43	11.50 ± 0.85	10.88 ± 0.72	1.75	0.197	1.52	0.228	0.28	0.604
Closed entries	7.50 ± 0.63	7.57 ± 0.57	7.17 ± 0.60	7.25 ± 0.80	0.01	0.910	0.23	0.633	0.01	0.933

hand, the animals repeatedly treated with solvent received either 2 mg/kg diazepam or solvent as a treatment.

2.4. Behavior in the EPM

The EPM apparatus was constructed of sheet metal, with a black rubber floor. It consisted of two opposed open arms (50 cm × 10 cm) with ledges (0.3 cm high) and two opposed enclosed arms (50 cm × 10 cm × 40 cm), connected by the junction area (10 cm × 10 cm). The whole apparatus was elevated 50 cm above the floor. Illumination in the experimental room was provided with one red neon tube fixed on the ceiling above the maze. Light intensity was 10 lx on the surface of the closed arms. Twenty minutes after administration of the appropriate treatment on the testing day, single rats were placed in the center of the maze, facing one of the enclosed arms and were allowed to freely explore the apparatus during 5 min. The standard spatiotemporal variables were recorded and analyzed using the ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA) in accordance with our previous studies (Savić et al., 2004, 2010). The primary indices of anxiety were the percentage of open arm entries, the percentage of time spent on the open arms and time spent in the distal parts of the open arms. The parameters closely related to motor activity were the total distance traveled, number of total entries and closed arm entries. An entry into an open arm, closed arm, or the distal part of the open arm was scored when 90% of animal crossed the virtual line separating the neighboring zones, whereas an exit occurred when more than 90% of animal left the respective zone. The distal part of open arms was defined as the area of the most distant 30% of open arms. Additionally, time spent in risk assessment behavior, defined as exiting a closed arm with forepaws and head only, and investigating the surroundings, was scored by an observer blind to treatment assignments. The risk assessment behavior represents a behavioral dimension closely related to certain aspects of anxiety (avoidance of danger, decision making and approach/avoid conflict) (Cruz et al., 1994; Rodgers and Johnson, 1995).

2.5. Statistical analysis

All numerical data presented in the figures were given as the mean ± SEM. In order to validate the experimental model of withdrawal-induced anxiety in our laboratory settings, a two-way ANOVA with treatment (withdrawal groups and control groups) and experimental condition (Experiment 1 and Experiment 2) as factors were applied. The effects of the antagonists, flumazenil and βCCt, on diazepam withdrawal-induced anxiety were analyzed by two separate one-way ANOVAs, in which withdrawal groups (WD) and acute diazepam groups (AD) served as reference groups for anxiogenic and anxiolytic response, respectively. Groups included in each statistical analysis were depicted in Fig. 1. If the ANOVA was significant ($P < 0.05$), *post hoc* Student–Newman–Keuls test (SNK's test) was performed. The animals that fell from the EPM were excluded from data analysis. Statistical analyses were performed with ANY-maze software, where applicable, while SigmaPlot 11.0 (Systat Software Inc., Richmond, CA, USA) was used elsewhere.

3. Results

3.1. The effects of diazepam withdrawal on behavior in the EPM

Table 1 shows the effects of diazepam withdrawal on the anxiety-related and motor activity-related parameters in the EPM. The two-way ANOVA, with treatment group and experimental condition as factors, revealed significant effect of treatment on the percentage of open time ($P = 0.023$). At the same time, decrease in

percentage of open arm entries and the time spent in the distal parts of the open arms for groups withdrawn from diazepam compared to control groups was close to significant ($P = 0.085$ and $P = 0.052$, respectively). Time spent in risk assessment behavior tended to be greater in groups withdrawn from diazepam ($P = 0.075$). The influence of treatment on the motor activity-related parameters (total distance traveled, total entries and closed arm entries) was not statistically significant. Neither experimental condition as factor nor interactions between factors were statistically significant.

3.2. Experiment 1

In regard to percentage of open time, the analysis showed a significant main effect of factor treatment ($F_{(2,18)} = 13.34$, $P < 0.001$). *Post hoc* SNK's test revealed that the percentage of open time was increased for groups WD + FLU and AD, in comparison with WD group ($P = 0.003$ and $P < 0.001$, respectively), as shown in Fig. 2A. The one-way ANOVA also revealed a significant main effect of treatment on the percentage of open arm entries ($F_{(2,18)} = 6.38$, $P = 0.008$). Animals from WD + FLU and AD groups showed significantly higher percentage of open arm entries than animals from WD group ($P = 0.028$ and $P = 0.007$, respectively), as shown in Fig. 2B. The influence of treatment on the time spent in the distal part of open arms was nearly significant ($F_{(2,18)} = 3,47$, $P = 0.053$) and *post hoc* SNK's test showed a significant difference between animals from AD and WD group ($P = 0.018$; Fig. 2C). Overall, there was a significant effect of treatment on the time spent in risk assessment behavior ($F_{(2,18)} = 12.48$, $P < 0.001$). *Post hoc* comparison revealed a significant decrease in this parameter for WD + FLU and AD groups compared to WD group ($P = 0.012$ and $P < 0.001$, respectively). Moreover, animals from WD + FLU group spent significantly more time in risk assessment behavior than animals from AD group ($P = 0.042$; Fig. 2D). In regard to motor activity-related parameters, one-way ANOVA did not reveal a significant influence of treatment on the total distance traveled ($F_{(2,18)} = 0.32$, $P = 0.729$) (data not shown) and total entries ($F_{(2,18)} = 0.16$, $P = 0.857$) (Fig. 2B). However, the influence of treatment on closed arm entries was significant, according to one-way ANOVA ($F_{(2,18)} = 4.84$, $P = 0.021$). *Post hoc* test revealed that animals from AD group exerted significantly fewer closed arm entries compared to animals from WD group ($P = 0.018$); this parameter could be observed in the Fig. 2B, as differences in total entries (gray bars) and open arm entries (hatched bars).

3.3. Experiment 2

Considering the percentage of open time, the one-way ANOVA demonstrated a significant influence of treatment ($F_{(4,34)} = 5.01$,
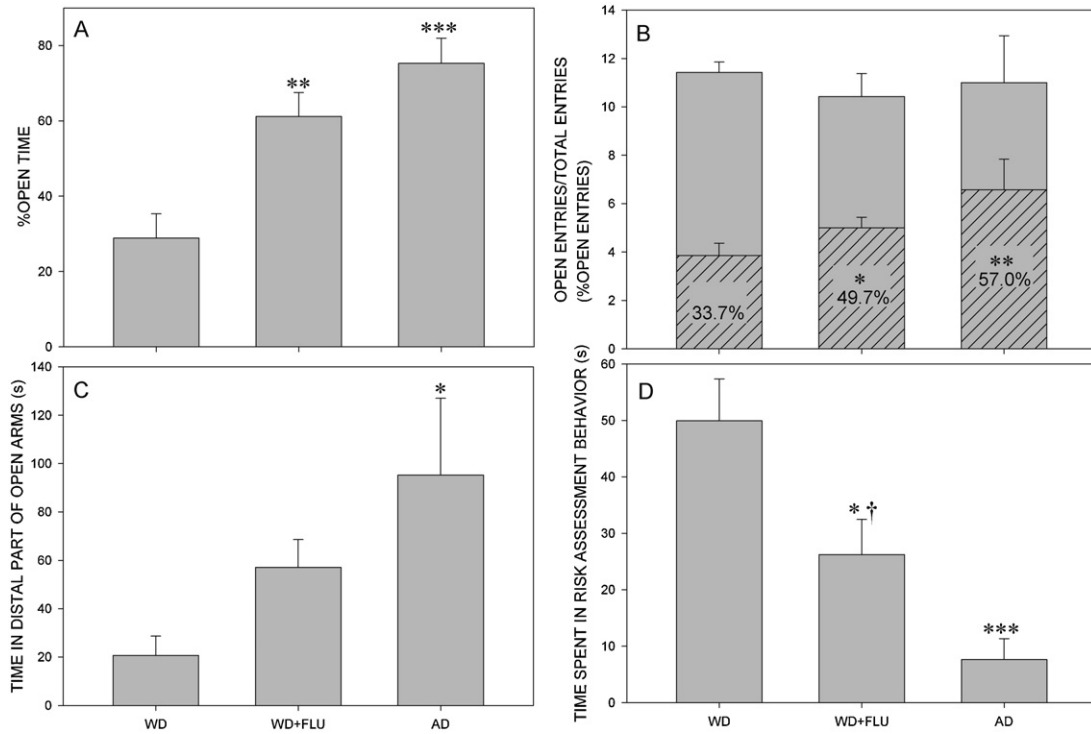
Fig. 2. The effects of diazepam withdrawal (WD), administration of 10 mg/kg flumazenil to diazepam withdrawn animals (WD + FLU) and acute administration of diazepam (AD) on the anxiety-related parameters. Graphs A, C and D represent the influence of treatment on the percentage of open time, the time spent in the distal parts of open arms and the time spent in risk assessment behavior, respectively. Graph B shows the effects of treatment on the percentage of open arm entries, indicated as the ratio of open arm entries (hatched bars) and total entries (open gray bars). *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the diazepam-withdrawn group (WD group); †P < 0.05 compared to the acute diazepam group (AD group). The number of animals per treatment group was 7.
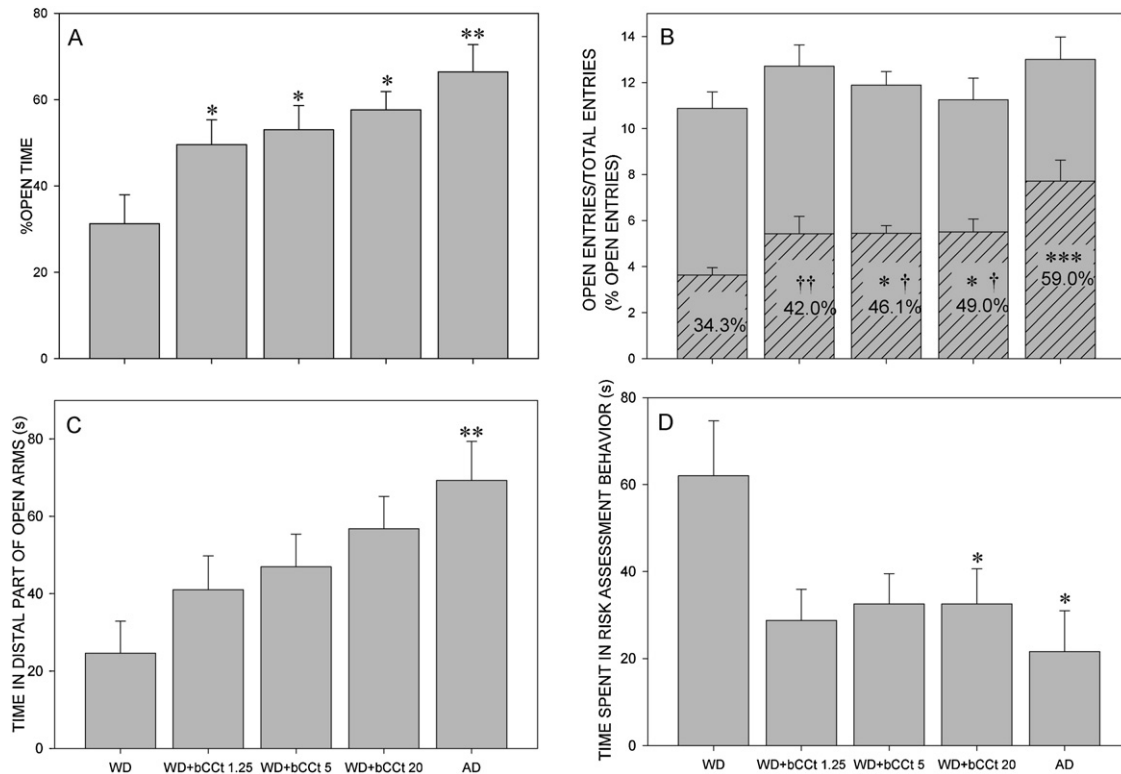

Fig. 3. The effects of diazepam withdrawal (WD), administration of 1.25 mg/kg, 5 mg/kg or 20 mg/kg βCCt to the diazepam withdrawn animals (WD + βCCt 1.25, WD + βCCt 5 and WD + βCCt 20) and acute administration of diazepam (AD) on the anxiety-related parameters. Graphs A, C and D represent the effects of treatment on the percentage of open time, the time spent in the distal parts of open arms and the time spent in risk assessment behavior. Graph B presents the effects of treatment on the percentage of open arm entries, indicated as the ratio of open arm entries (hatched bars) and total entries (open gray bars). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the diazepam-withdrawn group (WD group); †P < 0.05 and ††P < 0.01 compared to the acute diazepam group (AD group). The number of animals per treatment groups was 8, 7, 9, 8 and 7, for WD, WD + βCCt 1.25, WD + βCCt 5, WD + βCCt 20 and AD group, respectively.

$P = 0.003$). *Post hoc* test showed that for all the three groups withdrawn from diazepam and challenged with βCCt (WD + 1.25 βCCt, WD + 5 βCCt and WD + 20 βCCt), as well as for AD group, the percentage of open time was increased compared to WD group ($P = 0.032$, $P = 0.031$, $P = 0.014$ and $P = 0.001$, respectively; Fig. 3A). The influence of treatment on the percentage of open arm entries was also significant ($F_{(4,34)} = 6.88$, $P < 0.001$). SNK's test revealed that groups WD + 5 βCCt, WD + 20 βCCt and AD showed significantly higher percentage of open arm entries compared to WD group ($P = 0.049$, $P = 0.021$ and $P < 0.001$, respectively). Simultaneously, the percentage of open arm entries was lower for all the three groups challenged with βCCt (WD + 1.25 βCCt, WD + 5 βCCt and WD + 20 βCCt) compared to AD group ($P = 0.007$, $P = 0.03$ and $P = 0.046$, respectively), as presented in Fig. 3B. The influence of treatment on the time spent in the distal part of open arms was significant ($F_{(4,34)} = 3.53$, $P = 0.016$) and the AD group was different compared to WD group ($P = 0.009$; Fig. 3C). In regard to the time spent in risk assessment behavior, there was also a significant effect of treatment ($F_{(4,34)} = 2.88$, $P = 0.037$). Animals from the groups WD + 1.25 βCCt and WD + 5 βCCt tended to have the decreased time spent in risk assessment behavior in comparison with WD group ($P = 0.067$ and $P = 0.072$, respectively), while decreases of this parameter reached statistical significance for the groups WD + 20 βCCt and AD ($P = 0.028$ and $P = 0.029$ in comparison with WD group, respectively; shown in Fig. 3D). There was no influence of treatment on the analyzed motor activity-related parameters, namely total distance traveled ($F_{(4,34)} = 0.76$, $P = 0.561$), total entries ($F_{(4,34)} = 1,18$, $P = 0.335$) and closed arm entries ($F_{(4,34)} = 2.02$, $P = 0.113$) (data not shown).

4. Discussion

The withdrawal syndrome that occurs after abrupt discontinuation of chronic drug use is usually characterized by the emergence of a negative emotional state involving anxiety (Koob and Volkow, 2010). The EPM is a valuable animal model to study anxiety-like behavior that occurs during benzodiazepine withdrawal in rodents (File and Hitchcott, 1990). Using this paradigm, File and Andrews (1991) showed that repeated IP administration of 2 mg/kg diazepam, once daily during 21 days, could induce an anxiogenic response, measured 24 h after the last dose of diazepam. In the present study, the similar pattern of anxiety-like responses in rats after diazepam withdrawal was observed in both experiments. Increased anxiety was indicated by the decrease in open arm exploration and nearly significant increase in the time spent in risk assessment behavior.

In order to validate the experimental model of withdrawal-induced anxiety, we opted to use a proper vehicle control in one, but not in the other experiment (Experiment 2 vs. Experiment 1). Namely, it is known that intraperitoneal saline injection in rodents affects core temperature and heart rate, indicating an acute stress response (Dilsaver and Majchrzak, 1990; Meijer et al., 2006), which may bias the outcome in an anxiety test. However, subtle differences between two experimental designs did not influence the animals' behavior in our study, as there were no significant differences for factor experimental condition in the two-way ANOVA analysis for any of the observed parameters.

It has been postulated that benzodiazepine withdrawal affects several neuronal circuits. A close interrelationship between glutamatergic, dopaminergic and GABAergic neurons in key brain regions involved in the development of dependence and expression of anxiety is exemplified by findings that activation of α_1GABA$_A$ receptors triggers synaptic plasticity in the mesolimbic dopamine pathway and mediates inhibition of glutamatergic projection neurons in basolateral amygdala (Heikkinen et al., 2009; McDonald and Mascagni, 2004; Tan et al., 2010). Moreover, withdrawal after protracted benzodiazepine treatment leads to down-regulation of α_1GABA$_A$ receptors and functional uncoupling between GABA- and benzodiazepine- binding sites (Hu and Ticku, 1994; Tietz et al., 1999). These data suggest that prolonged diazepam treatment and subsequent withdrawal may modulate neuronal circuits by adaptations at α_1GABA$_A$ receptors. A major role of these receptors in processes underlying dependence liability has been supported by findings from pharmacological studies. Namely, SL651498, L-838, 417 and TPA 023, the compounds which bind to and modulate the benzodiazepine-sensitive GABA$_A$ receptors, but are devoid of efficacy at the α_1 subtype, are less prone to induce dependency (Ator et al., 2010; Griebel et al., 2003).

The results from Experiment 1 are in agreement with the finding that flumazenil is able to reverse the anxiety induced by withdrawal of diazepam in rats (File and Hitchcott, 1990). In the present study, acute challenge with flumazenil resulted in a significant increase of the percentages of open arm time and entries, as well as decrease of the time spent in risk assessment, compared to the rats subjected to withdrawal, but without challenge. Moreover, acute challenge with flumazenil induced an anxiolytic-like effect close, though not identical, to that seen with acute administration of diazepam. On the other hand, some previous reports indicated that flumazenil may precipitate a withdrawal syndrome in animals and humans (File and Hitchcott, 1990; Kaminski et al., 2003; Mintzer et al., 1999). File and Hitchcott (1990) suggested that such precipitation of withdrawal with flumazenil may be expected when tolerance to the anxiolytic effects of benzodiazepines has not yet developed and when subsequent anxiogenic response to drug withdrawal on its own does not ensue. Although the tolerance to the anxiolytic effect has not been investigated in the present study, the observed anxiogenic response during withdrawal could be seen as predictive of flumazenil's anxiolytic effect. Having in mind the data on adaptive changes at α_1GABA$_A$ receptors in key brain regions during withdrawal (reviewed in Uusi-Oukari and Korpi, 2010), and the propensity of flumazenil to rapidly reverse the GABA subsensitivity and the decreased α_1-subunit protein expression (Gallager et al., 1984; Tietz et al., 1999), we hypothesize that the antagonist's ability to act through α_1GABA$_A$ receptors may be responsible for the observed anxiolytic-like effect.

In Experiment 2, βCCt, a 20-fold selective antagonist at α_1GABA$_A$ receptors (Cox et al., 1995), was employed to further investigate the contribution of these receptors to the processes underlying the emergence of the increased anxiety during diazepam withdrawal. The interpretation of the data is critically dependent on the degree to which the in vitro selectivity of βCCt is reflected in vivo. In mice dosed with 30 mg/kg of βCCt, the binding reduction of radiolabeled flumazenil followed the relative distribution of the α_1-subunit, which revealed retained selectivity of βCCt binding (Griebel et al., 1999). Nevertheless, there was a study in mice suggesting that βCCt was able to antagonize the anxiolytic-like effects of chlordiazepoxide (Belzung et al., 2000), which are thought to be dominantly mediated by non-α_1GABA$_A$ receptors (Smith and Rudolph, 2012). However, chlordiazepoxide significantly increased motor activity in the given experimental conditions in the EPM (Belzung et al., 2000), and such a finding is known to have a potential to confound the data interpretation, sometimes leading to 'false positive' results (Dawson and Tricklebank, 1995). On the other hand, in an experiment in rats, βCCt failed to antagonize the anxiolytic effect of chlordiazepoxide (Carroll et al., 2001). This suggests existence of species differences between rats and mice, which may contribute to explanation of discrepancies between the reported (Belzung et al., 2000) and present results and indirectly support that βCCt was selective for α_1GABA$_A$ receptors in vivo in rats.

Acute challenge with βCCt, 24 h after the last diazepam injection, led to a significant reduction of diazepam withdrawal-induced anxiety. The influence of βCCt on the anxiety-related parameters in the EPM was partly affected by the dose administered. Namely, while acute challenge with any of the three doses of βCCt (1.25, 5 and 20 mg/kg) significantly increased the percentage of open time compared to the diazepam-withdrawn group, only administration of the two higher doses significantly increased the percentage of open arm entries, whereas only the dose of 20 mg/kg induced a significant decrease in the time spent in risk assessment behavior. While the influence of acute challenge with βCCt on anxiety-related parameters during withdrawal was comparable, with subtle differences between parameters, to the effects of the non-selective antagonist flumazenil, it could be observed that the anxiolytic-like effects of acute administration of diazepam tended in both experiments to be somewhat more pronounced than after acute challenge with two antagonists. On the whole, these data imply that GABA$_A$ receptors in brain were altered 24 h after termination of the protracted diazepam treatment, becoming susceptible to modulation by βCCt and flumazenil in a manner akin to the action of diazepam in experimentally naive rats. This hypothesis could be connected with findings of a decrease of α_1-containing GABA$_A$ receptors in certain rodent brain regions, 24 h after diazepam withdrawal (Impagnatiello et al., 1996).

In summary, the present study confirmed that abrupt cessation of protracted once-daily IP administration of 2 mg/kg diazepam could induce a withdrawal syndrome in rats, measured by increased anxiety-like behavior in the EPM paradigm 24 h after treatment cessation. Additionally, the study provided evidence that acute challenge with both, a non-selective antagonist flumazenil and a preferential α_1-subunit selective antagonist βCCt, alleviates the diazepam withdrawal-induced anxiety, and even induces anxiolytic-like response in rats withdrawn from diazepam. These findings suggest a common mechanism by which flumazenil and βCCt may reverse the withdrawal-induced anxiety. In order to further elucidate the mechanism of alleviation of the benzodiazepine withdrawal syndrome, future research should be directed to the downstream changes induced by long-term activation and acute antagonism at GABA$_A$ receptors containing the α_1 subunit. Finally, a closer examination of dose–response relationships with βCCt would be one of prerequisites needed for possible clinical testing of this selective antagonist following the withdrawal of benzodiazepines.

Acknowledgements

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