Research report

Duration of treatment and activation of α_1-containing GABA$_A$ receptors variably affect the level of anxiety and seizure susceptibility after diazepam withdrawal in rats

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ABSTRACT

Long-term use of benzodiazepine-type drugs may lead to physical dependence, manifested by withdrawal syndrome after abrupt cessation of treatment. The aim of the present study was to investigate the influence of duration of treatment, as well as the role of α_1-containing GABA$_A$ receptors, in development of physical dependence to diazepam, assessed through the level of anxiety and susceptibility to pentylenetetrazole (PTZ)-induced seizures, 24 h after withdrawal from protracted treatment in rats. Withdrawal of 2 mg/kg diazepam after 28, but not after 14 or 21 days of administration led to an anxiety-like behavior in the elevated plus maze. Antagonism of the diazepam effects at α_1-containing GABA$_A$ receptors, achieved by daily administration of the neutral modulator βCCt (5 mg/kg), did not affect the anxiety level during withdrawal. An increased susceptibility to PTZ-induced seizures was observed during diazepam withdrawal after 21 and 28 days of treatment. Daily co-administration of βCCt further decreased the PTZ-seizure threshold after 21 days of treatment, whilst it prevented the diazepam withdrawal-elicited decrease of the PTZ threshold after 28 days of treatment. In conclusion, the current study suggests that the role of α_1-containing GABA$_A$ receptors in mediating the development of physical dependence may vary based on the effect being studied and duration of protracted treatment. Moreover, the present data supports previous findings that the lack of activity at α_1-containing GABA$_A$ receptors is not sufficient to eliminate physical dependence liability of ligands of the benzodiazepine type.

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1. Introduction

Benzodiazepines were introduced into clinical practice more than 50 years ago and are still commonly prescribed drugs due to their anxiolytic, sedative-hypnotic, anticonvulsant and muscle relaxant effects. These drugs bind to a specific allosteric binding site at GABA$_A$ receptors and modulate activity of GABA$_A$ receptors that contain either of four α subunits: α_1, α_2, α_3, or α_5, in combination with the γ_2 subunit (Smith and Rudolph, 2012). However, despite clinical benefits of acute therapy, prolonged use of benzodiazepines is hampered by the development of tolerance to some of their behavioral effects, as well as physical dependence liability (Bateson,

2002). Physical dependence is a state of adaptation manifested by withdrawal syndrome: time-limited biochemical, physiological and behavioral disruptions upon termination of a regimen of chronic drug administration (Carter and Griffiths, 2009). Benzodiazepine withdrawal signs and symptoms range from mild to severe and may include anxiety, insomnia, irritability, headache, gastrointestinal disturbance, depersonalization and seizures (Griffiths and Weerts, 1997).

In the laboratory conditions, physical dependence to a benzodiazepine-type drug is assessed by the occurrence of the characteristic withdrawal signs upon cessation of the drug after protracted treatment (Licata and Rowlett, 2008). An anxiety-like behavior developed after benzodiazepine withdrawal in rats is commonly seen in the elevated plus maze test 12–96 h upon abrupt drug discontinuation (Izzo et al., 2001; dos Santos et al., 2010). Additionally, an enhanced susceptibility to seizures induced by

benzodiazepine partial inverse agonist (FG 7142), full inverse agonist (DMCM) or GABA$_A$ receptor antagonist – pentylenetetrazole (PTZ) in diazepam withdrawn animals has been shown to reflect the withdrawal hyperexcitability (Izzo et al., 2001; Mori et al., 2012; Tsuda et al., 1998). Although assessment of benzodiazepine dependence is conceptually simple, interpretation and comparison of the results from different laboratories can be complicated due to differences in duration of treatment, frequency of administration, the dose taken, the route of administration, time over which withdrawal syndrome will be observed, and choice of the behavioral response assessed (Ator and Griffiths, 2003; Moser et al., 2011).

The neurobiological mechanisms underlying dependence on benzodiazepine-type drugs are not well characterized yet. However, it is generally accepted that multiple adaptive mechanisms are involved in the different components of BZ dependence. In addition to the regionally and temporally selective changes at the level of GABA$_A$ receptor function (Pratt et al., 1998; Wright et al., 2014), the role of adaptive processes at excitatory synapses has been also indicated (Allison and Pratt, 2006). Close neuroanatomical relationships between glutamatergic and GABAergic neurons and the pivotal role of glutamatergic neurotransmission in mediating various forms of synaptic plasticity make glutamate a key candidate for changes in excitatory mechanisms and benzodiazepine dependence (Stephens, 1995; reviewed in Allison and Pratt, 2003). It has been shown that even a single injection of benzodiazepines is sufficient to induce synaptic plasticity at excitatory glutamatergic synapses, and such an initial adaptive step depended on the activation of α_1-containing GABA$_A$ receptors expressed on nearby interneurons (Heikkinen et al., 2009; Tan et al., 2010). The potentiation of α_1-containing GABA$_A$ receptors, expressed on interneurons, selectively inhibits GABA neurons and modulates glutamatergic neurotransmission, leading to the effect similar to that obtained with allopregnanolone and activation of presynaptic GABA$_A$ receptors (Iwata et al., 2013).

In pharmacological terms, studies on the contribution of specific subtypes of GABA$_A$ receptors in processes underlying initiation, development and manifestation of dependence to benzodiazepines are of particular interest. In this regard, it has been recently proposed that α_1-containing GABA$_A$ receptors play an important role in behaviors that are related to the initiation of physical dependence, following single administration of conventional benzodiazepines in squirrel monkeys (Fischer et al., 2013). Additionally, using different subtype selective positive allosteric modulators with reduced efficacy, Ator et al. (2010) and Mirza and Nielsen (2006) have shown that lack of efficacy at the α_1-containing GABA$_A$ receptors, probably coupled with the reduced efficacy at the α_2- and α_3-containing receptors, led to reduced physical dependence liability in baboons and mice, respectively. Moreover, a recent study from our group has demonstrated the significant involvement of α_1-containing GABA$_A$ receptors in the manifestation of increased anxiety during withdrawal (Divljaković et al., 2013).

In the present study, we investigated the influence of duration of treatment, as well as the role of α_1-containing GABA$_A$ receptors, in development of physical dependence to diazepam, assessed through the level of anxiety and susceptibility to PTZ-induced seizures, 24 h after withdrawal from diazepam protracted treatment in rats. To accomplish this, we used an innovative approach in the dependence research field. The α_1-selective neutral modulator βCCt (Huang et al., 1999) was protractedly co-administered with diazepam for 14, 21 or 28 days, in order to selectively block the diazepam's effects mediated through this GABA$_A$ receptor subtype. We aimed to compare the consequences of withdrawal from diazepam itself with those effects of this standard benzodiazepine ligand exerted through $\alpha_{2/3}$- or α_5-, but not α_1-containing GABA$_A$ receptors.

2. Materials and methods

2.1. Animals

Experiments were carried out on ninety male Wistar rats (Military Farm, Belgrade, Serbia), weighing 180–220 g at the beginning of experiment. 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 housed in plastic cages, five animals per cage, on a 12 h light/dark cycle (light on at 06:00 h). The temperature of the animal room was $22 \pm 1 ^\circ$C, the relative humidity 40–70%, the illumination 120 lx. Animals had free access to water and were fed with an amount of pellet food adequate to prevent excessive weight gain (Voss et al., 2003). Handling and testing took place during the light portion of the cycle.

2.2. Chemicals

The compounds used in the study were diazepam (Galenika, Serbia) and the preferentially α_1-subunit selective GABA$_A$ neutral modulator βCCt (t-butyl-β-carboline-3-carboxylate), synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, USA. These compounds were suspended in a solvent containing 85% distilled water, 14% propylene glycol, and 1% Tween 80, and were administered intraperitoneally (i.p.) in a volume of 1 ml/kg. PTZ (Sigma–Aldrich Chemie GmbH, Germany) was dissolved in physiological saline to prepare 40 mg/ml concentration for intravenous (i.v.) administration.

2.3. Experimental procedure

At the beginning of experiment, animals were randomly divided into nine pretreatment groups. In order to ensure that all animals passed through the same experimental procedure, rats were protractedly treated simultaneously with two injections of solvent (SOL–SOL), or 2 mg/kg diazepam and solvent (DZP–SOL), or 2 mg/kg diazepam and 5 mg/kg βCCt (DZP–βCCt), intraperitoneally (i.p.), once daily (at 8:30 h), during 14, 21 or 28 days. Approximately 24 h after the last administration of treatment, animals were tested in the elevated plus maze (EPM) test, followed by intravenous PTZ infusion test.

2.4. EPM test

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. 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; Divljaković et al., 2012). 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 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.

2.5. PTZ test

Immediately following EPM test, a single rat was placed in the Plexiglas box ($22 \times 10 \times 10\,cm^3$) with holes for ventilation, without strain. A butterfly infusion needle (needle size 25 G, ¾ in.) attached to a 20 ml syringe prefilled with PTZ solution was inserted into lateral tail vein. Correct placement was verified by the appearance of blood in the infusion tubing. PTZ was infused at a constant rate of 0.5 ml/min using a motor driven infusion pump (Stoelting Co., Wood Dale Illinois, USA). The volume of solution infused before the appearance of full-blown clonic-tonic seizure was recorded (Fahey et al., 2006). The threshold doses of PTZ (mg/kg) required to elicit seizures were calculated from the volume of infusion (ml), concentration of PTZ (mg/ml) and body weight (kg), using the following formula: volume of PTZ (ml) × concentration of PTZ (mg/ml)/body weight (kg).

2.6. Data analysis

All numerical data presented in the figures were given as the mean ± SEM. Data obtained from the EPM test and PTZ test were analyzed using two-way ANOVA, with treatment and duration of treatment as factors. If the ANOVA showed significant effects of factors or interaction ($p < 0.05$), *post hoc* Newman–Keuls test was performed. Animals that fell from EPM apparatus and those which did not develop seizure due to improper cannulation of the tail vein were omitted 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. EPM test

In regard to motor activity-related parameters (Fig. 1), two-way ANOVA revealed a significant influence of factor duration of treatment on the total distance traveled ($F_{(2,74)} = 4.59$, $P = 0.013$), total arm entries ($F_{(2,74)} = 3.27$, $P = 0.044$) and closed arm entries ($F_{(2,74)} = 3.70$, $P = 0.030$). The influences of factor treatment and interaction between factors were not significant for these three parameters (total distance traveled: treatment $F_{(2,74)} = 2.64$, $P = 0.078$ and interaction $F_{(4,74)} = 0.26$; $P = 0.903$; total arm entries: treatment $F_{(2,74)} = 2.40$, $P = 0.098$ and interaction $F_{(4,74)} = 0.44$, $P = 0.781$; closed arm entries: treatment $F_{(2,74)} = 3.08$, $P = 0.052$ and interaction $F_{(4,74)} = 0.18$, $P = 0.949$). *Post hoc* test revealed that rats protractedly treated for 21 days have shown significantly higher total distance traveled (Fig. 1a) than animals protractedly treated during 14 and 28 days ($P = 0.015$ and $P = 0.020$, respectively). The parameter total arm entries (Fig. 1b) was higher for animals treated for 21 days than for those treated for 28 days ($P = 0.031$); the closed arm entries (Fig. 1c) was higher for groups treated for 21 days than for 14-days treated groups ($P = 0.028$).

Anxiety-related parameters are represented in Fig. 2. Two-way ANOVA did not reveal a significant influence of factors treatment or duration of treatment on any of the three parameters, namely the percentage of time on open arms (treatment $F_{(2,74)} = 0.76$, $P = 0.472$ and duration $F_{(2,74)} = 1.17$, $P = 0.316$); the percentage of open arm entries (treatment $F_{(2,74)} = 0.84$, $P = 0.435$ and duration $F_{(2,74)} = 1.70$, $P = 0.190$); and the time in distal part of open arms (treatment
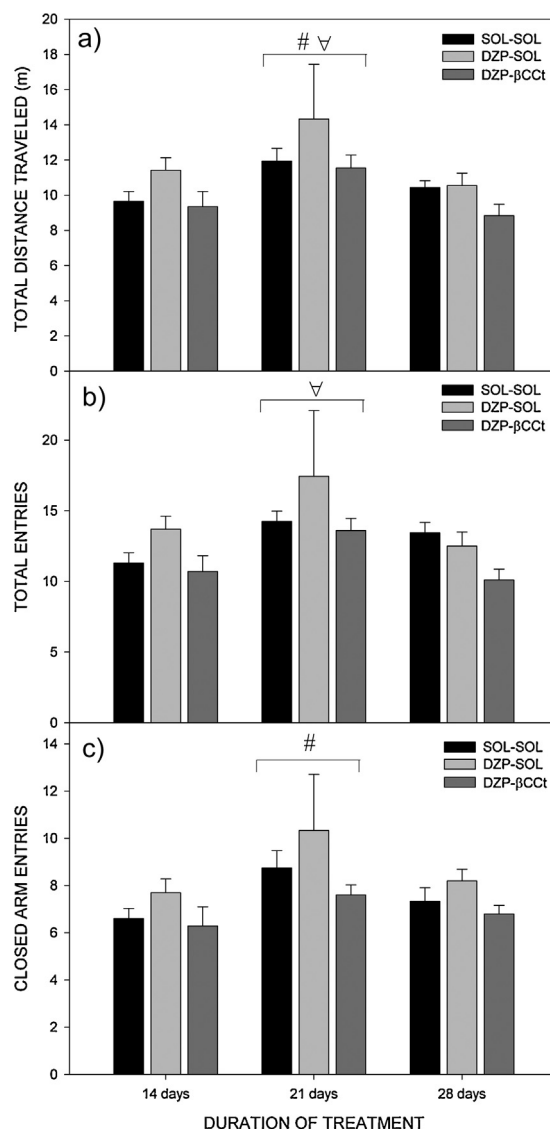

Fig. 1. The influence of withdrawal from protracted treatment for 14, 21 and 28 days with solvent (SOL–SOL), 2 mg/kg diazepam (DZP–SOL) and 2 mg/kg diazepam with 5 mg/kg βCCt (DZP–βCCt) on the motor activity-related parameters in the EPM test. Graphs (a)–(c) represent the effects of treatment and duration of treatment on the total distance traveled, total arm entries and closed arm entries, respectively. #$P < 0.05$ compared to 14 days of treatment, ∀ $P < 0.05$ compared to 28 days of treatment. Number of animals per group were: after 14 days of protracted treatment, 10, 10 and 7; after 21 days of treatment, 8, 9 and 10; after 28 days of treatment, 9, 10 and 10, for SOL–SOL, DZP–SOL and DZP–βCCt groups, respectively.

$F_{(2,74)} = 0.17$, $P = 0.846$ and duration $F_{(2,74)} = 2.90$, $P = 0.061$). Interaction between factors was significant for the percentage of time on open arms ($F_{(4,74)} = 3.13$, $P = 0.020$, Fig. 2a), while it was insignificant for the percentage of open arm entries and time spent in distal part of open arms ($F_{(4,74)} = 2.23$, $P = 0.074$ and $F_{(4,74)} = 1.62$, $P = 0.179$, respectively; Fig. 2b and c). In regard to percentage of time on open arms, *post hoc* test revealed that animals withdrawn from protracted treatment with diazepam or diazepam and βCCt for 28 days have shown significantly lower percentage of time in open arms than the group protractedly treated with solvent ($P = 0.027$ and $P = 0.018$, respectively).

3.2. PTZ test

Results from PTZ test are represented in Fig. 3. Two-way ANOVA revealed a significant effect of factor treatment ($F_{(2,63)} = 12.01$,
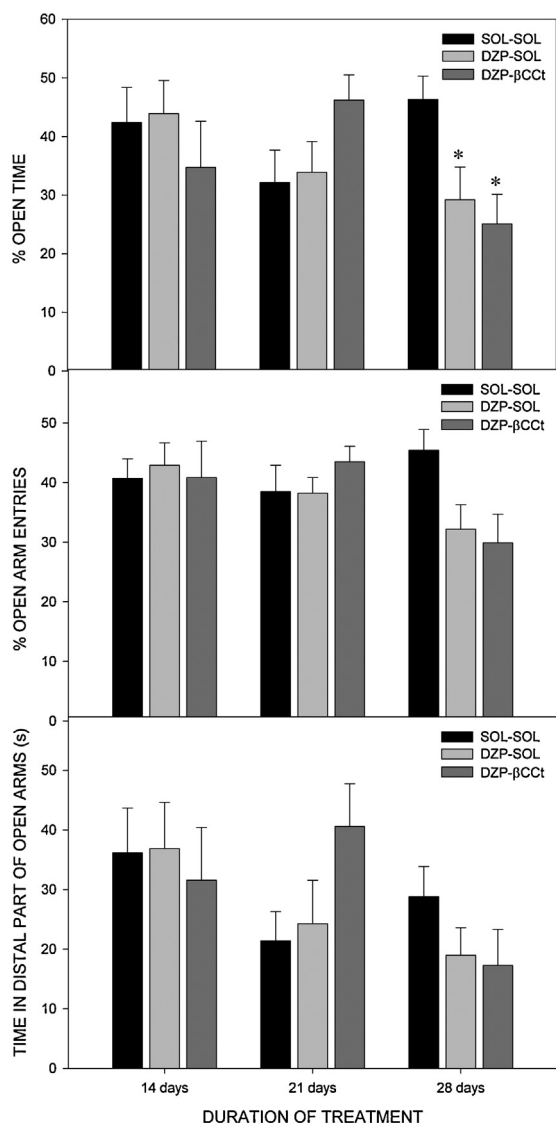
Fig. 2. The influence of withdrawal from protracted treatment for 14, 21 and 28 days with solvent (SOL–SOL), 2 mg/kg diazepam (DZP–SOL) and 2 mg/kg diazepam with 5 mg/kg βCCt (DZP–βCCt) on the anxiety-related parameters in the EPM test. Graphs (a)–(c) represent the effects of treatment and duration of treatment on the percentage of time on open arms, the percentage of open arm entries and time spent in distal part of open arms. * *P* < 0.05 compared to respective SOL–SOL group.
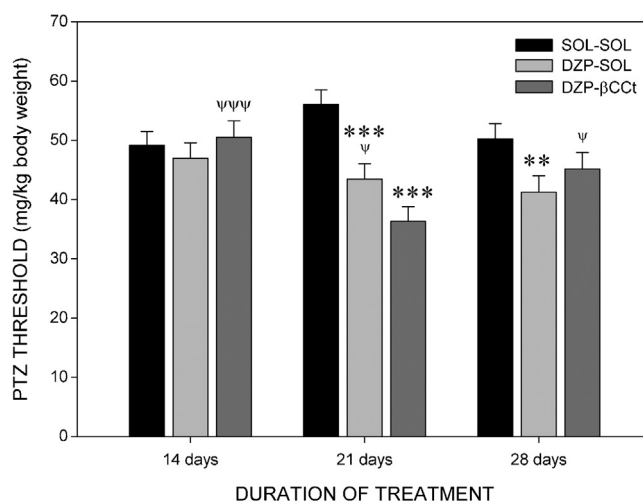

Fig. 3. The influence of withdrawal from protracted treatment for 14, 21 and 28 days with solvent (SOL–SOL), 2 mg/kg diazepam (DZP–SOL) and 2 mg/kg diazepam with 5 mg/kg βCCt (DZP–βCCt) on the threshold dose of PTZ in the PTZ infusion test. ** *P* < 0.01, *** *P* < 0.001 compared to respective SOL–SOL group, ψ *P* < 0.05, ψψψ *P* < 0.001 compared to DZP–βCCt after 21 days. Number of animals per group were: after 14 days of protracted treatment, 10, 8 and 7; after 21 days of treatment, 9, 8 and 7; and after 28 days of treatment, 8, 7 and 7, for SOL–SOL, DZP–SOL and DZP–βCCt groups, respectively.

4. Discussion

Development of physical dependence of benzodiazepine-type drugs can be affected by the complex interaction between several factors, such as the administered dose, dosing frequency, route of administration and duration of chronic treatment (Ator and Griffiths, 2003; Moser et al., 2011), which altogether govern receptor exposure (Mirza and Nielsen, 2006). In this study we showed that withdrawal of 2 mg/kg diazepam for 24 h after 28 days of protracted treatment led to an anxiety-like behavior in the EPM. An increased susceptibility to PTZ-induced seizures was observed during the diazepam withdrawal after 21 and 28 days of protracted treatment. Antagonism of the diazepam effects at α_1-containing GABA$_A$ receptors throughout the protracted treatment, achieved by co-administration of the neutral modulator βCCt at a dose of 5 mg/kg, did not influence the anxiety level during withdrawal. In regard to seizure susceptibility, protracted co-administration of βCCt with diazepam further decreased the PTZ-seizure threshold after 21 days of treatment, whilst it prevented the diazepam withdrawal-elicited decrease of the PTZ threshold after 28 days of treatment.

Selective antagonism of the specific subtype of GABA$_A$ receptors, as the innovative pharmacological approach in the investigations of dependence liability, offers additional benefits of time-limited blockade during the chosen period of time, but is critically dependent on the pharmacokinetic characteristics of the ligands. For diazepam, elimination half-lives from plasma and brain (18.24 ± 1.00 min and 28.18 ± 6.70 min, respectively) obtained in our unpublished study (Savić et al., unpublished data) have confirmed its fast elimination following a single i.p. dose in rats (Friedman et al., 1986). Nevertheless, repeated administration of diazepam once daily for 7 days can still result in significant accumulation of the parent molecule as well as the metabolite N-methyldiazepam in rats (Fernandes et al., 1996). In accordance with the suggested longer duration of action of βCCt (Zhang et al., 1995; June et al., 2003), its brain concentration of about 55 nmol/kg, measured 16 h after the last dose in protracted (twice daily for 14 days) administration of βCCt with diazepam (Savić et al., unpublished data), implies that βCCt in the present work was sufficient

P < 0.001), whilst the influence of factor duration of treatment was not significant ($F_{(2,63)} = 2.58$, *P* = 0.084). There was a significant interaction between factors ($F_{(4,63)} = 5.91$, *P* < 0.001). After 14 days of treatment, *post hoc* test did not reveal significant differences in the threshold dose of PTZ between treatment groups. On the other hand, withdrawal after 21 days of protracted treatment with diazepam or diazepam and βCCt led to significant decrease of the threshold dose of PTZ, 24 h after the last treatment injection (*P* < 0.001 for both treatments, compared to SOL–SOL group); the decrease of PTZ threshold dose was more prominent for DZP–βCCt group than for DZP–SOL (*P* = 0.040). Newman–Keuls test also demonstrated a significant decrease of the PTZ threshold dose for DZP–SOL group in comparison with SOL–SOL group, 24 h after 28 days of treatment (*P* = 0.007). Finally, significantly higher doses of PTZ were needed to induce seizures 24 h after protracted treatment with diazepam and βCCt for 14 days and 28 days in comparison to the threshold dose needed after 21 days of the same treatment (*P* < 0.001 and *P* = 0.015, respectively).

to prevent binding of diazepam at α_1-containing GABA$_A$ receptors, without affecting the withdrawal behavior. Finally, it is noteworthy that in the current study the compounds were dissolved in the same solvent as used in our previous behavioral and pharmacokinetic studies (Divljaković et al., 2012, 2013; Savić et al., unpublished data).

In contrast to previous results (Izzo et al., 2001; Begg et al., 2005; dos Santos et al., 2010), the present study did not indicate development of physical dependence after discontinuation of diazepam treatment for 14 days. Namely, we did not observe either an increased susceptibility to PTZ-induced seizures or an increased level of anxiety 24 h after the last administration of 2 mg/kg diazepam. As the present treatment schedule comprised administration of a relatively low diazepam dose, at low dosing frequency and by the route of administration characterized by fast elimination, the reason for the apparent discrepancy could be an insufficient exposure to the drug. This finding supports the claims on safety and efficacy of benzodiazepines prescribed in low doses for 1 or 2 weeks only, or intermittently (Ashton, 1994).

In the present EPM experiment, a stimulation of locomotion was observed for all groups treated for 21 days in comparison to the groups treated for 14 and 28 days, indicating an adaptive behavioral response to the environmental conditions and everyday handling (Costa et al., 2012; Schmitt and Hiemke, 1998). Such an adaptive behavioral response was not observed in our previous studies, when the protracted i.p. administration of treatment has been also applied (Divljaković et al., 2012, 2013). The subtle differences in the experimental design, such as co-administration of two injections in the present, instead of one injection in previous studies, could have favored the development of the increased locomotion after 21 days of treatment. Noteworthy, the EPM test may yield a false positive anxiolytic-like effect when the procedure or treatment increases locomotor activity (Dawson and Tricklebank, 1995). Hence, the detected hyperlocomotion could at least partly account for the observed discordance between current results from the EPM after 21 days of treatment and our previous finding, when 24 h after discontinuation of the same diazepam treatment an anxiogenic-like response was observed (Divljaković et al., 2013). Furthermore, a trend of increase of the motor activity-related parameters ($p < 0.1$) was more prominent for the group withdrawn from diazepam treatment than for other two groups after 21 days of treatment. Arguably, this finding could be explained by a mild diazepam withdrawal-induced response, in a manner similar to the previously described with zolpidem (Elliot and White, 2000).

An overt anxiety-like response after diazepam withdrawal, in the absence of significant changes in motor activity, was detected after 28 days of protracted treatment. Co-administration of diazepam with βCCt for 28 days did not change anxiety behavior during withdrawal, suggesting that protracted blockade of α_1-containing GABA$_A$ receptors does not suffice to prevent the development of an increased level of anxiety during diazepam withdrawal. However, acute challenge with βCCt in a dose range from 1.25 to 20 mg/kg alleviated the anxiety induced by abrupt cessation of protracted treatment with diazepam (Divljaković et al., 2013). Taken together, these data suggest that non-α_1 GABA$_A$ receptors-mediated mechanisms are essential for induction of withdrawal-induced anxiety, whilst acute blockade of α_1-containing GABA$_A$ receptors may be sufficient for reduction of such anxiety.

The PTZ test showed increased seizure susceptibility after diazepam withdrawal after 21 days and 28 days of protracted treatment. Our findings obtained after 21, but not 28 days of protracted treatment may be seen as concordant with the hypothesis that animals more sensitive to convulsions might be less prone to anxiety (Chapouthier and Venault, 2001). On the other hand, the present study implies that the PTZ test is more sensitive and suitable in

detecting effects of the benzodiazepine withdrawal than the EPM test. It also suggests that results from PTZ test are not affected by daily manipulation of animals. The finding that withdrawal after 21 days of repeated co-administration of diazepam and βCCt has further increased susceptibility to the PTZ-induced seizures may mean that activation of α_1-containing GABA$_A$ receptors during protracted administration of diazepam exerts an alleviating effect on development of increased seizure susceptibility during withdrawal. However, this finding is in contrast with results after 28 days of protracted treatment in the present study, when antagonism of the effects mediated through the activation of α_1-containing GABA$_A$ receptors, achieved with co-administration of diazepam and βCCt, showed the ability to prevent the increased seizure susceptibility during withdrawal.

The observed differences in the withdrawal effects after 21 and 28 days of protracted treatment, as well as differences between findings from EPM and PTZ test could be the consequence of differential adaptive changes in the neuroanatomical circuits, as suggested by Pratt et al. (1998). Furthermore, the differential involvement of α_1-containing GABA$_A$ receptors in the mechanisms underlying benzodiazepine physical dependence could be hypothesized from conflicting findings with zolpidem, the preferentially α_1- selective positive allosteric modulator of GABA$_A$ receptors (Sanna et al., 2002). Namely, the decreased dependence liability of zolpidem after protracted treatment in mice (Elliot and White, 2000; Mirza and Nielsen, 2006; Perrault et al., 1992) supports our data from the EPM test after 28 days and PTZ test after 21 days, and points to the role of non-α_1-containing GABA$_A$ receptors in the development of physical dependence, or even an alleviating effect of the activation of α_1-containing GABA$_A$ receptors in this process. On the other hand, findings of withdrawal syndrome after discontinuation of zolpidem in primates (Weerts et al., 1998), but also in mice (Wright et al., 2014), support our data from PTZ test after 28 days, suggesting that activation of α_1-containing GABA$_A$ receptors is necessary for development of phenomena related with protracted administration of benzodiazepines. Taken together, antagonism at α_1-containing GABA$_A$ receptors is probably insufficient for consistent prevention of the development of physical dependence, while α_1-sparing ligands coupled with reduced activity at non-α_1-containing GABA$_A$ receptors might be candidates suitable for long-term therapy (Ator et al., 2010; Mirza and Nielsen, 2006).

In conclusion, the current study suggests that the role of predominant α_1-containing GABA$_A$ receptors in mediating development of physical dependence relates to the observed effect and duration of protracted benzodiazepine treatment. Hence, it may be not possible to identify one particular subtype of GABA$_A$ receptors as being responsible for the development and expression of physical dependence, and further efforts aimed at developing ligands devoid of physical dependence liability should be pointed toward reduction of activity at non-α_1-containing GABA$_A$ receptors.

References

Allison, C., Pratt, J.A., 2003. Neuroadaptive processes in GABAergic and glutamatergic systems in benzodiazepine dependence. Pharmacol. Ther. 98, 171–195.

Allison, C., Pratt, J.A., 2006. Differential effects of two chronic diazepam treatment regimes on withdrawal anxiety and AMPA receptor characteristics. Neuropsychopharmacology 31, 602–619.

Ashton, H., 1994. Guidelines for the rational use of benzodiazepines. When and what to use. Drugs 48, 25–40.

Ator, N.A., Atack, J.R., Hargreaves, R.J., Burns, H.D., Dawson, G.R., 2010. Reducing abuse liability of GABAA/benzodiazepine ligands via selective partial agonist

efficacy at alpha 1 and alpha 2/3 subtypes. J. Pharmacol. Exp. Ther. 332, 4–16.

Ator, N.A., Griffiths, R.R., 2003. Principles of drug abuse liability assessment in laboratory animals. Drug Alcohol Depend. 70, S55–S72.

Bateson, A.N., 2002. Basic pharmacologic mechanisms involved in benzodiazepine tolerance and withdrawal. Curr. Pharm. Des. 8, 5–21.

Begg, D.P., Hallam, K.T., Norman, T.R., 2005. Attenuation of benzodiazepine withdrawal anxiety in the rat by serotonin antagonists. Behav. Brain Res. 161, 286–290.

Carter, L.P., Griffiths, R.R., 2009. Principles of laboratory assessment of drug abuse liability and implications for clinical development. Drug Alcohol Depend. 105, S14–S25.

Chapouthier, G., Venault, P., 2001. A pharmacological link between epilepsy and anxiety? Trends Pharmacol. Sci. 22, 491–493.

Costa, R., Tamascia, M.L., Nogueira, M.D., Casarini, D.E., Marcondes, F.K., 2012. Handling of adolescent rats improves learning and memory and decreases anxiety. J. Am. Assoc. Lab. Anim. Sci. 51, 548–553.

Dawson, G.R., Tricklebank, M.D., 1995. Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol. Sci. 16, 33–36.

Divljaković, J., Milić, M., Namjoshi, O.A., Tiruveedhula, V.V., Timić, T., Cook, J.M., Savić, M.M., 2013. βCCT, an antagonist selective for α(1)GABA(A) receptors, reverses diazepam withdrawal-induced anxiety in rats. Brain Res. Bull. 91, 1–7.

Divljaković, J., Milić, M., Timić, T., Savić, M.M., 2012. Tolerance liability of diazepam is dependent on the dose used for protracted treatment. Pharmacol. Rep. 64, 1116–1125.

dos Santos, L., de Andrade, T.G., Graeff, F.G., 2010. Social separation and diazepam withdrawal increase anxiety in the elevated plus-maze and serotonin turnover in the median raphe and hippocampus. J. Psychopharmacol. 24, 725–731.

Elliot, E.E., White, J.M., 2000. Precipitated and spontaneous withdrawal following administration of lorazepam but not zolpidem. Pharmacol. Biochem. Behav. 66, 361–369.

Fahey, J.M., Grassi, J.M., Reddi, J.M., Greenblatt, D.J., 2006. Acute zolpidem administration produces pharmacodynamic and receptor occupancy changes at similar doses. Pharmacol. Biochem. Behav. 83, 21–27.

Fernandes, C., File, S.E., Berry, D., 1996. Evidence against oppositional and pharmacokinetic mechanisms of tolerance to diazepam's sedative effects. Brain Res. 734, 236–242.

Friedman, H., Abernethy, D.R., Greenblatt, D.J., Shader, R.I., 1986. The pharmacokinetics of diazepam and desmethyldiazepam in rat brain and plasma. Psychopharmacology (Berlin) 88, 267–270.

Fischer, B.D., Teixeira, L.P., van Linn, M.L., Namjoshi, O.A., Cook, J.M., Rowlett, J.K., 2013. Role of gamma-aminobutyric acid type A (GABAA) receptor subtypes in acute benzodiazepine physical dependence-like effects: evidence from squirrel monkeys responding under a schedule of food presentation. Psychopharmacology (Berlin) 227, 347–354.

Griffiths, R.R., Weerts, E.M., 1997. Benzodiazepine self-administration in humans and laboratory animals – implications for problems of long-term use and abuse. Psychopharmacology (Berlin) 134, 1–37.

Heikkinen, A.E., Möykkynen, T.P., Korpi, E.R., 2009. Long-lasting modulation of glutamatergic transmission in VTA dopamine neurons after a single dose of benzodiazepine agonists. Neuropsychopharmacology 34, 290–298.

Huang, Q., Cox, E.D., Gan, T., Ma, C., Bennett, D.W., McKernan, R.M., Cook, J.M., 1999. Studies of molecular pharmacophore/receptor models for GABAA/benzodiazepine receptor subtypes: binding affinities of substituted beta-carbolines at recombinant alpha × beta 3 gamma 2 subtypes and quantitative structure–activity relationship studies via a comparative molecular field analysis. Drug Des. Discov. 16, 55–76.

Iwata, S., Wakita, M., Shin, M.C., Fukuda, A., Akaike, N., 2013. Modulation of allopregnanolone on excitatory transmitters release from single glutamatergic terminal. Brain Res. Bull. 93, 39–46.

Izzo, E., Auta, J., Impagnatiello, F., Pesold, C., Guidotti, A., Costa, E., 2001. Glutamic acid decarboxylase and glutamate receptor changes during tolerance and dependence to benzodiazepines. Proc. Natl. Acad. Sci. U. S. A. 98, 3483–3488.

June, H.L., Foster, K.L., McKay, P.F., Seyoum, R., Woods, J.E., Harvey, S.C., Eiler, W.J., Grey, C., Carroll, M.R., McCane, S., et al., 2003. The reinforcing properties of alcohol are mediated by GABA(A1) receptors in the ventral pallidum. Neuropsychopharmacology 28, 2124–2137.

Licata, S.C., Rowlett, J.K., 2008. Abuse and dependence liability of benzodiazepine-type drugs: GABA(A) receptor modulation and beyond. Pharmacol. Biochem. Behav. 90, 74–89.

Mirza, N.R., Nielsen, E.Ø., 2006. Do subtype-selective gamma-aminobutyric acid A receptor modulators have a reduced propensity to induce physical dependence in mice? J. Pharmacol. Exp. Ther. 316, 1378–1385.

Mori, T., Shimizu, N., Shibasaki, M., Suzuki, T., 2012. Involvement of the arachidonic acid cascade in the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal. Biol. Pharm. Bull. 35, 2243–2246.

Moser, P., Wolinsky, T., Duxon, M., Porsolt, R.D., 2011. How good are current approaches to nonclinical evaluation of abuse and dependence? J. Pharmacol. Exp. Ther. 336, 588–595.

Perrault, G., Morel, E., Sanger, D.J., Zivkovic, B., 1992. Lack of tolerance and physical dependence upon repeated treatment with the novel hypnotic zolpidem. J. Pharmacol. Exp. Ther. 263, 298–303.

Pratt, J.A., Brett, R.R., Laurie, D.J., 1998. Benzodiazepine dependence: from neural circuits to gene expression. Pharmacol. Biochem. Behav. 59, 925–934.

Sanna, E., Busonero, F., Talani, G., Carta, M., Massa, F., Peis, M., Maciocco, E., Biggio, G., 2002. Comparison of the effects of zaleplon, zolpidem, and triazolam at various GABA(A) receptor subtypes. Eur. J. Pharmacol. 451, 103–110.

Savić, M.M., Obradović, D.I., Ugrešić, N.D., Cook, J.M., Yin, W., Bokonjić, D.R., 2004. Bidirectional effects of benzodiazepine binding site ligands in the elevated plus-maze: differential antagonism by flumazenil and beta-CCt. Pharmacol. Biochem. Behav. 79, 279–290.

Schmitt, U., Hiemke, C., 1998. Strain differences in open-field and elevated plus-maze behavior of rats without and with pretest handling. Pharmacol. Biochem. Behav. 59, 807–811.

Smith, K.S., Rudolph, U., 2012. Anxiety and depression: mouse genetics and pharmacological approaches to the role of GABA(A) receptor subtypes. Neuropharmacology 62, 54–62.

Stephens, D.N., 1995. A glutamatergic hypothesis of drug dependence: extrapolations from benzodiazepine receptor ligands. Behav. Pharmacol. 6, 425–446.

Tan, K.R., Brown, M., Labouèbe, G., Yvon, C., Creton, C., Fritschy, J.M., Rudolph, U., Lüscher, C., 2010. Neural bases for addictive properties of benzodiazepines. Nature 463, 769–774.

Tsuda, M., Shimizu, N., Yajima, Y., Suzuki, T., Misawa, M., 1998. Hypersusceptibility to DMCM-induced seizures during diazepam withdrawal in mice: evidence for upregulation of NMDA receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 357, 309–315.

Voss, J., Sanchez, C., Michelsen, S., Ebert, B., 2003. Rotarod studies in the rat of the GABA(A) receptor agonist gaboxadol: lack of ethanol potentiation and benzodiazepine cross-tolerance. Eur. J. Pharmacol. 482, 215–222.

Weerts, E.M., Ator, N.A., Grech, D.M., Griffiths, R.R., 1998. Zolpidem physical dependence assessed across increasing doses under a once-daily dosing regimen in baboons. J. Pharmacol. Exp. Ther. 285, 41–53.

Wright, B.T., Gluszek, C.F., Heldt, S.A., 2014. The effects of repeated zolpidem treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with diazepam. Psychopharmacology (Berlin) [Epub ahead of print].

Zhang, W., Koehler, K.F., Zhang, P., Cook, J.M., 1995. Development of a comprehensive pharmacophore model for the benzodiazepine receptor. Drug Des. Discov. 12, 193–248.