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ORIGINAL INVESTIGATION

Insights into functional pharmacology of α_1 $GABA_A$ receptors: how much does partial activation at the benzodiazepine site matter?

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Abstract
Rationale Synthesis of ligands inactive or with low activity at α_1 $GABA_A$ receptors has become the key concept for development of novel, more tolerable benzodiazepine (BZ)-like drugs. WYS8, a remarkably (105 times) α_1-subtype selective partial positive modulator, may serve as a pharmacological tool for refining the role of α_1 $GABA_A$ receptors in mediation of BZs' effects.
Objectives Here, the effects of WYS8 on GABA-induced currents and on diazepam-induced potentiation of recombinant BZ-sensitive $GABA_A$ receptors were studied in more detail. In addition, the behavioral profile of WYS8 (0.2, 1, and 10 mg/kg i.p.), on its own and in combination with diazepam, was tested in the spontaneous locomotor activity, elevated plus maze, grip strength, rotarod, and pentylenetetrazole tests.
Results WYS8, applied at an in vivo attainable concentration of 100 nM, reduced the stimulation of GABA currents by 1 μM diazepam by 57 % at $\alpha_1\beta_3\gamma_2$, but not at $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, or $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors. The administration of WYS8 alone induced negligible behavioral consequences. When combined with diazepam, WYS8 caused a reduction in sedation, muscle relaxation, and anticonvulsant activity, as compared with this BZ alone, whereas ataxia was preserved, and the anxiolytic effect of 2 mg/kg diazepam was unmasked.
Conclusions Hence, a partial instead of full activation at α_1 $GABA_A$ receptors did not necessarily result in the attenuation of the effects assumed to be mediated by activation of these receptors, or in the full preservation of the effects mediated by activation of other $GABA_A$ receptors. Thus, the role of α_1 $GABA_A$ receptors appears more complex than that proposed by genetic studies.

Keywords Recombinant receptors · Efficacy · Anxiolytic · Sedation · Ataxia · Muscle relaxation · Anticonvulsant

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Introduction

The last two decades have witnessed numerous breakthroughs in understanding the mechanisms of action of psychopharmacologic drugs, which were originally introduced into clinical practice mainly without clear-cut knowledge of the intricate receptor substrates underlying their pharmacological actions (Fibiger 2012). From the applicative point of view, the final goal of these efforts would be to introduce novel, receptor-selective ligands which clinically separate wanted from unwanted effects. The research field of the benzodiazepines (BZs), positive allosteric modulators of the BZ binding site at $GABA_A$ receptors in the central nervous system, has been thought of as especially promising in this regard. Namely, these widely used drugs clinically act through no more than four major $GABA_A$ receptor populations, which contain the α_1, α_2, α_3, or α_5 subunit in addition to a β and the γ_2 subunit (Chang et al. 1996) and have distinct patterns of anatomical distribution in the brain (Sieghart and Ernst 2005). Thus, it was logical to hypothesize that each of the receptor subtypes may mediate a

distinct set of BZ effects, allowing synthesis of ligands which modulate only receptor(s) involved in clinically wanted actions (Dawson et al. 2005).

The studies with point-mutated mice, made insensitive to diazepam at only one of four populations of BZ-sensitive $GABA_A$ receptors, have attributed the sedative and ataxic properties of this standard BZ to the α_1 subunit-containing $GABA_A$ receptor, the anxiolytic and muscle relaxant actions to the α_2/α_3 subtype-containing receptors, and anticonvulsant activity, in part, to all the $\alpha_1/\alpha_2/\alpha_3$ $GABA_A$ receptors (reviewed in Rudolph and Möhler 2004). Based on these results, inactivity/low-activity at α_1 $GABA_A$ receptors has evolved as the key concept for development of new BZ-like drugs, purportedly devoid of many adverse effects of standard BZs, such as sedation or abuse liability (Dawson et al. 2005; Rudolph and Knoflach 2011). However, generalization of this concept in the quest for anxioselective anxiolytics has been questioned by the findings in humans that several ligands, reportedly possessing properties of either null or low efficacy at α_1 $GABA_A$ receptors, still exerted the effects reflective of sedation (reviewed in Skolnick 2012). These failures in clinical development have tempered original enthusiasm, clearly demanding refinement in the concept of preclinical research.

While it is perceivable that inactivation of a whole receptor population by a point mutation is suitable for the identification of main effects of one receptor subtype, such an approach cannot be expected to allow the identification of a possible subtle modulation of behavioral effects primarily mediated by other receptors (cf. Morris et al. 2006). However, it could be possible to observe such subtle modulation when the effects of diazepam mediated by certain receptor subtypes are not nullified by point mutations but only inhibited by selective antagonists. The effects of BZs mediated by α_1 $GABA_A$ receptors have been studied using pharmacological manipulation with the approximately 20 times α_1-subunit selective neutral modulator (antagonist) β-CCt (t-butyl-β-carboline-3-carboxylate) (Rowlett et al. 2005). In addition, recently we have demonstrated that WYS8 (8-ethynyl-β-carboline-3-carboxylate-t-butyl ester), a close congener of β-CCt with even higher selectivity in affinity for α_1 $GABA_A$ receptors (Yin et al. 2010), behaves as a weak partial positive modulator (Joksimović et al. 2013). When combined with diazepam, the in vivo actions of these two close congeners were clearly differentiable: β-CCt proved to be an antagonist of diazepam's effects (Savić et al. 2009), while WYS8 not only did not antagonize but even potentiated some of diazepam's effects in the water maze (Joksimović et al. 2013).

Here, we present a wider set of electrophysiological data on WYS8 and also report on the in vitro selectivity in its antagonism of diazepam's effects exerted through α_1-containing but not other BZ-sensitive $GABA_A$ receptors. Furthermore, the present study investigated WYS8 on its own and in combination with diazepam in behavioral procedures designed to assess the sedative, anxiolytic, muscle relaxant, ataxic, and anticonvulsant effects of positive modulators at the BZ binding site with the aid of the spontaneous locomotor activity, elevated plus maze, grip strength, rotarod, and pentylenetetrazole tests, respectively.

Experimental procedures

Drugs

The novel compound WYS8 (8-ethynyl-ß-carboline-3-carboxylate-t-butyl ester; 8-ethynyl-ß-CCt) was synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee. For electrophysiological study, diazepam was purchased from Sigma-Aldrich, St. Louis, MO, USA while, for behavioral experiments, diazepam was obtained from Galenika (Belgrade, Serbia).

Two electrode voltage clamp

cDNAs of rat $GABA_A$ receptor subunits were used for generating the respective mRNA's that were then injected into *Xenopus laevis* oocytes (Nasco, WI) as described previously (Ramerstorfer et al. 2010). For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of *Xenopus* Ringer solution (XR, containing 90 mM NaCl, 5 mM HEPES–NaOH (pH 7.4), 1 mM $MgCl_2$, 1 mM KCl, and 1 mM $CaCl_2$). The oocytes were constantly washed by a flow of 6 ml/min XR which could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from dimethyl sulfoxide (DMSO)-solutions resulting in a final concentration of 0.1 % DMSO perfusing the oocytes. Drugs were preapplied for 30 s before the addition of GABA, which was coapplied with the drugs until a peak response was observed. Between two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. To test for modulation of GABA-induced currents by drugs, a concentration of GABA was used that was titrated to trigger 3 % of the respective maximum GABA-elicited current of the individual oocyte (EC_3). At this low GABA concentration, within- and between-day currents are reproducible and effects of drugs (percent of modulation) are much higher than that at higher GABA concentrations, contributing to the accuracy of the results. In addition, such low GABA concentrations correspond to those acting at extrasynaptic receptors that represent the majority of $GABA_A$ receptors in the brain (Kasugai et al., 2010). In contrast, GABA concentrations acting at synaptic $GABA_A$ receptors in many cases fully activate these receptors. At such GABA concentrations, benzodiazepines cannot enhance GABA currents anymore, but they

prolong current decay. This effect, however, is antagonized by a simultaneously occurring rapid receptor desensitization making measurements unreliable. The GABA EC_3 concentration was applied to the cell together with various concentrations of drugs. For current measurements, the oocytes were impaled with two microelectrodes (2–3 mΩ) which were filled with 2 mM KCl. All recordings were performed at room temperature at a holding potential of −60 mV using a Warner OC-725C two-electrode voltage clamp (Warner Instruments, Hamden, CT). Data were digitized, recorded, and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA). Results of concentration response experiments were graphed using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA). Data were graphed as mean±SEM of at least four oocytes from at least two batches.

In addition to experiments performed previously (Joksimović et al. 2013), the presently performed experiments aimed to obtain: (1) concentration effect curves for WYS8 at recombinant $\alpha_4\beta_3\gamma_2$ and $\alpha_6\beta_3\gamma_2$, as well as at $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors; (2) electrophysiological recordings for 1 μM diazepam and 10 nM, 100 nM, and 1 μM WYS8, on their own and in respective combinations at $\alpha_1\beta_3\gamma_2GABA_A$ receptors, and for 1 μM diazepam and 100 nM WYS8, on their own and in combination at $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, and $\alpha_5\beta_3\gamma_2GABA_A$ receptors.

Behavioral experiments

Experiments were carried out on male Wistar rats (Military Farm, Belgrade, Serbia), weighing 220–250 g. 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 transparent plastic cages, six animals per cage, and had free access to food pellets and tap water. The temperature of the animal room was 22±1 °C, the relative humidity 40–70 %, the illumination 120 lux, and the 12/12 h light/dark period (light on at 6:00 h). All handling and testing took place during the light phase of the diurnal cycle (testing started at 9:00 h). Separate groups of animals were used for all behavioral paradigms, with the exception of the grip strength and pentylenetetrazole test, which were consecutively performed in the same animals. The rats' behavior in the spontaneous locomotor activity and the elevated plus maze was recorded by a ceiling-mounted camera and analyzed by the ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA). In the spontaneous locomotor activity test, grip strength, and pentylenetetrazole test, we used eight treatment groups altogether: solvent, diazepam (2 mg/kg), WYS8 (0.2, 1, and 10 mg/kg), diazepam (2 mg/kg)+WYS8 (0.2, 1, and 10 mg/kg). In the elevated plus maze and rotarod, we added four more groups: diazepam (1 mg/kg) and diazepam (1 mg/kg)+WYS8 (0.2, 1, and 10 mg/kg) or diazepam (0.5 mg/kg) and diazepam (0.5 mg/kg)+WYS8 (0.2, 1, and 10 mg/kg), respectively. The ligands 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 an overall volume of 2 ml/kg, 20 min before behavioral testing.

Measurement of locomotor activity

Twenty minutes after receiving the appropriate treatment, single rats were placed in a clear Plexiglas chamber (40×25×35 cm). Activity under dim red light (20 lux) was recorded for a total of 30 min, without any habituation period. Besides the total distance traveled, behavior was analyzed by dividing the locomotor activity data into 5-min bins.

Behavior in 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 a junction area (central platform) measured 10×10 cm. A ledge of sheet metal (0.3 cm high) surrounding the open arms was added. The illumination in the experimental room consisted of one red neon tube fixed on the ceiling, giving light intensity of 10 lux on the surface of the closed arms. At the beginning of the experiment, single rats were placed in the center of the maze, facing one of the enclosed arms, and their behavior was recorded for 5 min. An entry into an open or closed arm was scored when 90 % of the animal crossed the virtual line separating the central square of the maze from the arm, whereas an exit occurred when more than 90 % of the animal left the respective arm. After each trial, the maze was cleaned with dry and wet towels.

Grip strength test

Muscle strength was assessed by the grip strength meter (Ugo Basile, Milan, Italy, model 47105). When pulled by the tail, the rat grasps the trapeze connected to a force transducer, and the apparatus measures the peak force of experimenter's pull (in grams) necessary to overcome the strength of the animal's forelimbs grip. Each animal was given three consecutive trials, and the median value of three readings was used for further statistics.

Rotarod performance

Rotarod test (Ugo Basile, Comerio, Italy) measured the capacity of the animal to maintain itself on the revolving rod. The

rats were trained for 4 days, with three 2-min trials each day. On the first training day, speed of revolutions was fixed at 15 rpm while, afterwards, an acceleration 15–25 rpm was imparted. In the morning of the fifth day, the final selection was made. Only animals capable of remaining 120 s on the rod, without any fall, were chosen for further testing. The rotarod performance was tested 20 min after treatment.

Pentylenetetrazole test

Pentylenetetrazole (70 mg/kg) was used as a standard chemical convulsant eliciting, according to our preliminary experiments, a characteristic epileptic-like syndrome in at least 80 % of rats. It was injected subcutaneously 20 min after the pretreatment with WYS8 and/or diazepam. The protection against clonic or tonic seizures during the 30-min observation period was considered as an index of anticonvulsant activity.

Statistical analysis

For electrophysiological data, the paired Student's *t* test was used, comparing the means of the percent of GABA EC_3 stimulation with a hypothetical value of 100 (100 % of GABA EC_3–control current). Data sets from behavioral studies were checked for homogeneity of variance and normality prior to analysis by a two-way ANOVA (four levels of factor WYS8 and two or three levels of factor diazepam). Where applicable, Student–Newman–Keuls's (SNK) test for post hoc comparisons was also used. In the case of significant interaction, separate one-way ANOVAs and Student's *t* tests were conducted to assess the influence of one factor within individual levels of other factor. In the pentylenetetrazole test, a chi-square test was performed to determine if there was overall difference in the frequency of incidence of clonic or tonic convulsions due to pretreatment with different doses of WYS8 and/or diazepam. Afterward, the two respective incidences were compared by Fisher's exact test. Statistical analyses were performed with ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA) and Sigma Plot 11 (Systat Software Inc., Richmond, CA, USA). Differences were considered to be statistically significant when p was less than 0.05, while $0.1>p>0.05$ was considered as a trend toward significance.

Results

Two-electrode voltage clamp

The in vitro concentration–effect curves presenting percent potentiation of a within- and between-day stable GABA EC_3 response for WYS8 at recombinant $\alpha_{1-6}\beta_3\gamma_2$ $GABA_A$ receptors, together with the comparison of its effects at $\alpha_1\beta_{1-3}\gamma_2$ receptors, are given in Fig. 1. Besides modulatory influences on α_1-, α_2-, α_3-, and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors, which have been published previously (Joksimović et al. 2013), here, we additionally report on the effects of WYS8 at BZ-insensitive α_4- and α_6-containing $GABA_A$ receptor subtypes. In a manner analogous to the actions of WYS8 at other non-α_1 $GABA_A$ receptors, no considerable modulation was reached at concentrations lower than 100 nM (α_4 $GABA_A$ receptors) or 10 μM (α_6 $GABA_A$ receptors) (Fig. 1). Potentiating actions at β_1-, β_2-, and β_3-containing α_1 $GABA_A$ receptors were similar to each other in a wide range of WYS8 concentrations (1 nM–1 μM) (inset of Fig. 1).

Electrophysiological behavior of diazepam (at 1 μM), WYS8 (at 100 nM), and their combinations is presented in Fig. 2, together with explicit data on percent potentiation of EC_3 GABA response at rat recombinant α_1-,α_2-, α_3-, and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors. In addition, Fig. 2 shows in vitro activity at $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors of 10 nM and 1 μM WYS8, on their own and in combination with 1 μM

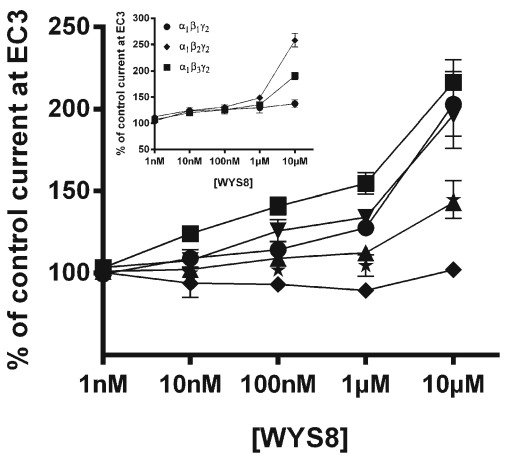

Fig. 1 Main figure: Concentration–effect curves for WYS8 on $\alpha_1\beta_3\gamma_2$ (*closed square*), $\alpha_2\beta_3\gamma_2$ (*triangle*), $\alpha_3\beta_3\gamma_2$ (*inverted triangle*), $\alpha_4\beta_3\gamma_2$ (*closed circle*), $\alpha_5\beta_3\gamma_2$ (*closed diamond*), and $\alpha_6\beta_3\gamma_2$ (*asterisk*) $GABA_A$ receptors, using an EC_3 GABA concentration. Data points in *main figure* and *inset* represent means±SEM from three to six oocytes from ≥2 batches. The percentages of stimulation for BZ-sensitive $GABA_A$ receptor subtypes have already been given in Joksimović et al. (2013). A significant potentiation of $\alpha_4\beta_3\gamma_2$ receptors was detected at 100 nM (114±3%), 1 μM (128±4%), and 10 μM (203±10%) of WYS8. At $\alpha_6\beta_3\gamma_2$ receptors, only the highest concentration of WYS8 resulted in a significant change relative to control current (145±5%). *Inset*: In separate experiments performed in parallel, a concentration of 10 nM of WYS8 resulted in 124±4%, 125±3%, and 120±2% of control current in $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$, and $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors, respectively. The tenfold higher concentration of WYS8 resulted in 126±5%, 131±4%, and 126±3% of control current in $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$, and $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors, respectively. All values given were significantly different from the respective control currents ($p<0.05$, one sample Student's *t* test)

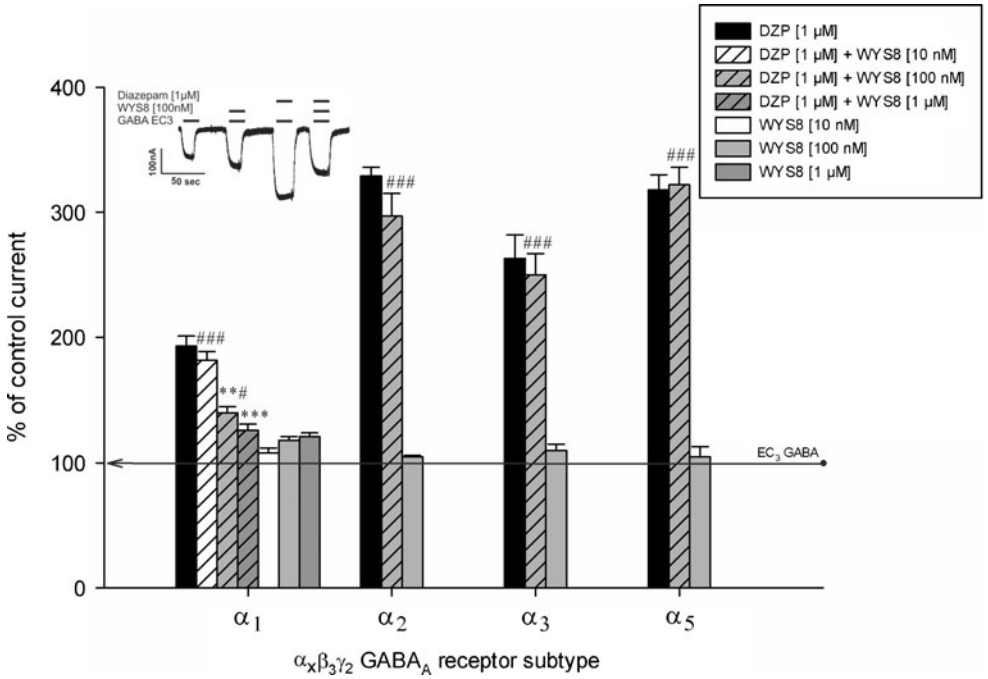

Fig. 2 *Bar graphs* indicating the effects of diazepam (DZP; 1 μM), WYS8 (10 nM, 100 nM, 1 μM) and their combination at $\alpha_x\beta_3\gamma_2$ $GABA_A$ receptors, using an EC_3 GABA concentration. Data points represent means+SEM from at least three (n=3–8) oocytes from ≥2 batches. The concentration of 1 μM of diazepam resulted in 193±8%, 329±7%, 263±19%, and 318±12% of control current at α_1-, α_2-, α_3-, and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors, respectively. The tested concentration of WYS8 (100 nM) reached significance only at α_1- and $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors (118±3% and 110±5%, respectively). The addition of WYS8 (100 nM) to diazepam (1 μM) resulted in 140±5%, 297±18%, 250± 17%, and 322±14% of control current at α_1-, α_2-, α_3-, and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors, respectively. The WYS8 (10 nM)+diazepam (1 μM) combination resulted in 182±7% of control current at $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. At the same receptor population, the addition of WYS8 (1 μM) to diazepam (1 μM) resulted in 126±5% of control current. $^{**}p<0.01$ and $^{***}p<0.001$ compared with DZP (1 μM); $\#p<0.05$ and $\#\#\#p<0.01$ compared with the respective concentration of WYS8. The *inset* shows examples of current traces of GABA EC_3, WYS8 (100 nM), diazepam (1 μM), and their combination at $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors

diazepam. While diazepam exerted significant potentiation of GABA EC_3 at all $GABA_A$ receptor subtypes, WYS8 behaved as a weak partially effective positive modulator at $\alpha_1\beta_3\gamma_2$ and produced a borderline significant effect at $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors; this pattern of activity was essentially similar to that already reported (Joksimović et al. 2013). The addition of WYS8 at concentration of 100 nM resulted in a significant reduction of diazepam's potentiation of only α_1-containing $GABA_A$ receptors. At these receptors, WYS8 also exerted a comparable attenuating effect at 1 μM but not 10 nM.

Motor activity assay

A two-way ANOVA applied on the analysis of the influence on total distance traveled in the activity chamber (Fig. 3) indicated a significant effect of the factor diazepam (F(1,48)=11.02, p=0.002), whereas the dose of the WYS8 as a factor was of borderline significance (F(3,48)=2.81, p=0.050). The diazepam×WYS8 interaction was not significant (F(3,55)=0.14, p=0.937). Post hoc SNK's method showed that there were no significant differences among single levels of the factor WYS8; the greatest difference of means (3.462 m) existed between doses of 0.2 and 10 mg/kg (p=0.054). On the other hand, the existing significant difference of means (3.087 m) between the two levels of diazepam (2 versus 0 mg/kg, p=0.002) was based on difference within WYS8 0 (4.109 m, p=0.032) and disappeared when multiple comparisons were made within the 0.2, 1, and 10 mg/kg WYS8 doses (respective p values 0.148, 0.128, and 0.166), showing that the sedative action of diazepam was attenuated by addition of WYS8.

When the two-way ANOVA for traveled distance was developed into 5-min intervals (Fig. 4), it turned out that the influence of diazepam as a factor was significant in the periods 0–5 min (F(1,48)=8.31, p=0.006) and 5–10 min (F(1,48)=22.99, p<0.001). While the sedative effect of diazepam in the first interval was based solely on the difference within the WYS8 0 level (difference of means 1.90 m, p=0.025), significant differences in the next interval (5–10 min) were present within the 0, 0.2, and 1 mg/kg WYS8 doses (respective differences of means 1.39 (p=0.036), 1.86 (p=0.06), and 1.67 m (p=0.013)); the difference within the 10 mg/kg dose of WYS8 was nearly significant (1.27 m, p=0.055). This analysis has shown that there existed a short period when the sedative action of

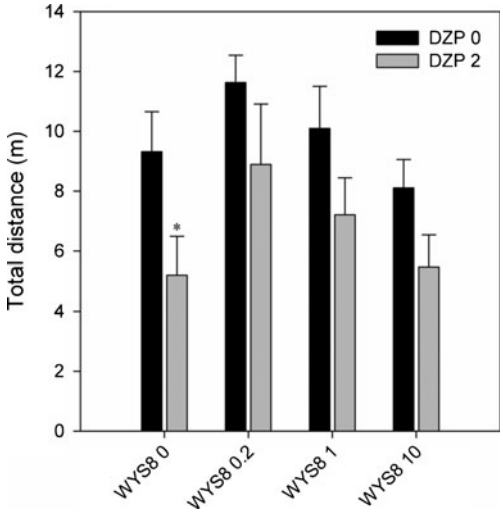

Fig. 3 The effects of WYS8 (0, 0.2, 1, and 10 mg/kg) in the absence (DZP 0) and the presence of 2 mg/kg diazepam (DZP 2), on the total distance traveled in the activity chamber during 30 min of recording. Data points represent means±SEM. *$p<0.05$ compared with DZP 0 within WYS8 0. Number of animals per each treatment was 7

diazepam was not amenable to amelioration by WYS8. On the other hand, the influence of the factor WYS8 was significant in the interval 10–15 min ($F(3,48)=3.42$, $p=0.024$). Post hoc comparison among single levels revealed the significant difference between the 0.2 and 10 mg/kg doses of WYS8 (difference of means 0.97 m, $p=0.015$). The significance was based on the difference within the diazepam (0 mg/kg) but not diazepam 2 mg/kg dose (respective differences of means 1.18 and 0.76 m), suggesting that in this interval the highest dose of WYS8 may have exerted a mild sedative-like action. The interaction between two factors did not reach significance at either of intervals.

Elevated plus maze

The effect of diazepam as a factor was statistically significant for both the activity-related parameters (for total distance (Fig. 5a): $F(2,72)=7.20$, $p=0.001$; for closed arm entries (Fig. 5b): $F(2,72)=7.61$, $p=0.001$) and the anxiety-related parameters (for percent of open arm time (Fig. 5c)—$F(2,72)=4.60$, $p=0.013$; for percent of open arm entries (Fig. 5d)—$F(2,72)=3.82$, $p=0.027$), as analyzed with a two-way ANOVA. The respective F values ($F(3,72)$) for WYS8 as a factor were, for activity-related parameters, $F=2.53$ ($p=0.064$) and $F=3.35$ ($p=0.024$) while, for anxiety-related parameters, $F=0.18$ ($p=0.91$) and $F=0.66$ ($p=0.577$). The interaction between two factors was not significant in either of the analyses.

For both activity-related parameters, post hoc comparisons revealed a significant overall difference in the influence of the 2 mg/kg diazepam versus 0 mg/kg as well as 1 mg/kg dose. As shown in the Fig. 4a, b, this difference existed within the 10 mg/kg dose of WYS8. Within the 1 mg/kg dose of WYS8, 2 mg/kg diazepam decreased the number of closed arm entries in comparison to the lower dose. Similarly, the dose of 10 mg/kg of WYS8 exerted a significant overall decrease of closed arm entries relative to the 0 and 1 mg/kg doses of WYS8. Within the 2 mg/kg diazepam level, the effect of the 10 mg/kg dose of WYS8 was significant when compared with each of three other levels of WYS8; the latter also applies to the parameter of the total distance. In regard to the anxiety-related parameters, 2 mg/kg diazepam significantly increased both, the percent of open arm time and percent of open arm entries in comparison with the control group; for the latter parameter, there was also difference between the 0 and 1 mg/kg dose of diazepam. As shown in Fig. 4d, there was also a significant increase of percent of open arm entries with 2 mg/kg diazepam when compared with 1 mg/kg but only within the 0.2 mg/kg dose of WYS8. Within this level of WYS8, there existed a clear statistical trend toward revealing the anxiolytic effect of diazepam, implied by an increase in both the percent of open arm time ($p=0.068$ for 2 versus 0 mg/kg diazepam and $p=0.061$ for 2 mg/kg versus 1 mg/kg diazepam) and the percent of open arm entries ($p=0.065$ for 2 versus 0 mg/kg diazepam).

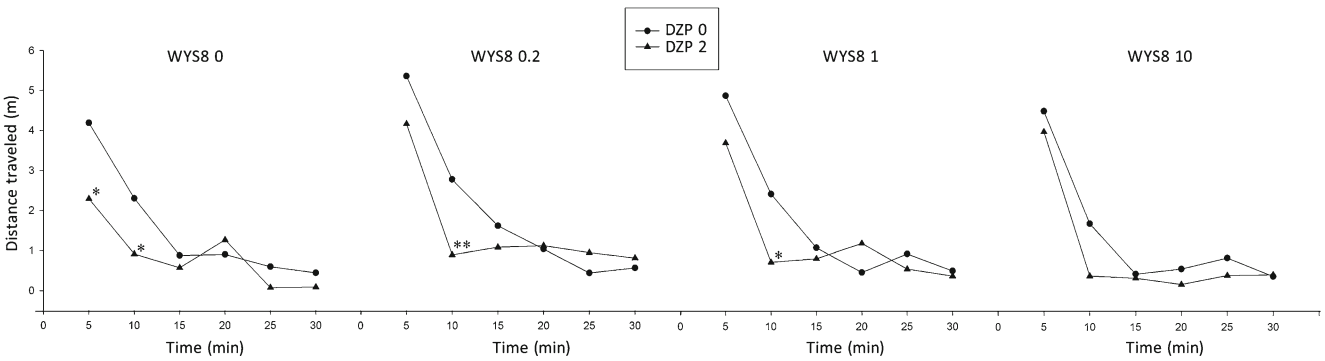

Fig. 4 The effects of WYS8 (0, 0.2, 1, and 10 mg/kg) in the absence (DZP 0; *closed circles*) and the presence of 2 mg/kg diazepam (DZP 2; *closed triangles*), on the distance traveled in the activity chamber during six consecutive 5-min intervals. Data points represent means. *$p<0.05$ and **$p<0.01$ compared with DZP 0 within the appropriate dose of WYS8

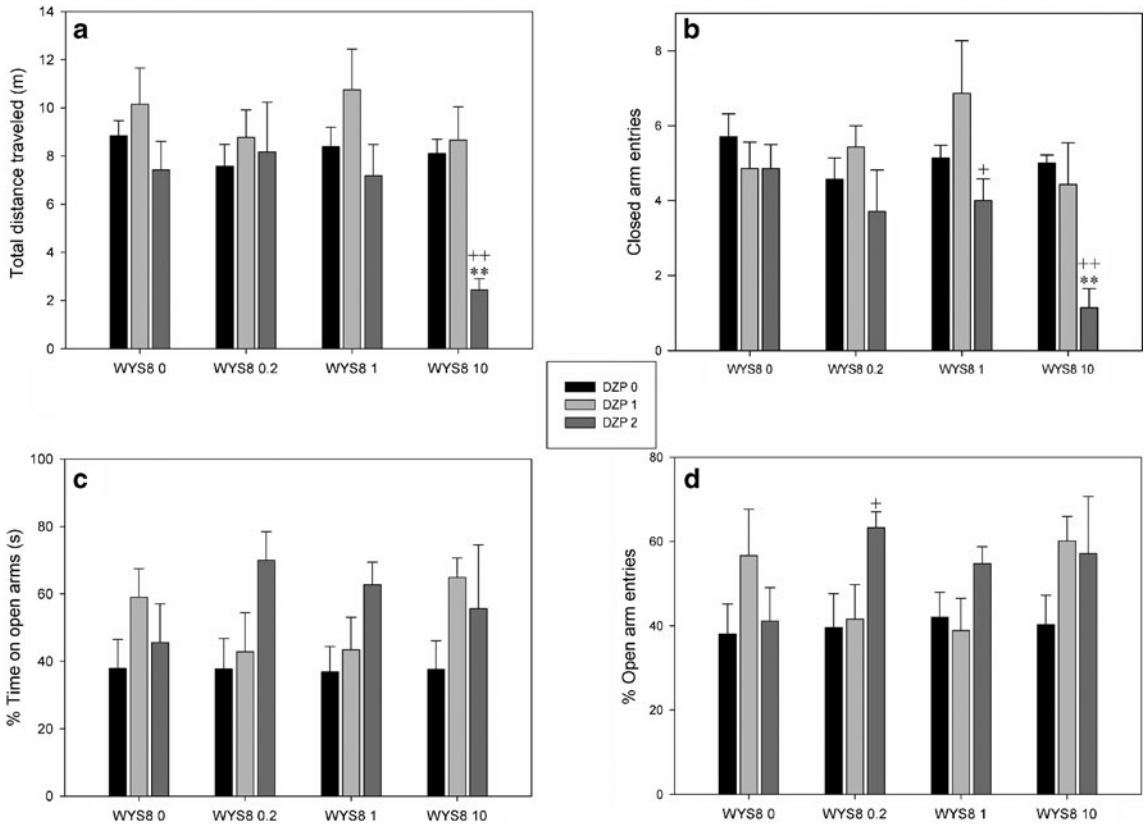

Fig. 5 The effects of diazepam (DZP; 0, 1, and 2 mg/kg) and WYS8 (0, 0.2, 1, and 10 mg/kg) on the **a** total distance traveled, **b** number of closed arm entries, **c** percentage of time spent on open arms, and **d** percentage of entries in open arms of the elevated plus maze. Data points represent means+SEM. $^{**}p<0.01$ compared with the DZP 0 group within the appropriate dose of WYS8; $^{+}p<0.05$ and $^{++}p<0.01$ compared with the DZP 1 group within the appropriate dose of WYS8. Number of animals per each treatment was 7

Grip strength

The two-way ANOVA of the results obtained in the grip strength test (Fig. 6) showed that both, diazepam ($F(1,45)=22.03$, $p<0.001$) and WYS8 ($F(3,45)=8.36$, $p<0.001$) exerted a highly significant influence. Since a significant interaction between diazepam and WYS8 was also determined ($F(3,45=10.35$, $p<0.001$), we decided to use one-way ANOVAs (for each of two levels of factor diazepam) and Student's *t* tests (for each of four levels of factor WYS8) to fully examine the simple main effects of both factors. One-way ANOVAs revealed the borderline significance of WYS8 effect within the factor diazepam 0 ($F(3,24)=2.999$, $p=0.050$) but a highly significant effect within diazepam 2 ($F(3,21)=15.412$, $p<0.001$). Post hoc analysis further revealed a significant difference between each level of WYS8, except for 10 versus 0.2 mg/kg WYS8 comparison. Student's *t* tests applied on diazepam data within different levels of WYS8 (0, 0.2, and 10 mg/kg, but not 1 mg/kg) showed a highly significant difference between the two levels of diazepam, revealing that the incapacitating effect of diazepam (2 mg/kg) was amenable to antagonism by WYS8 only at 1 mg/kg.

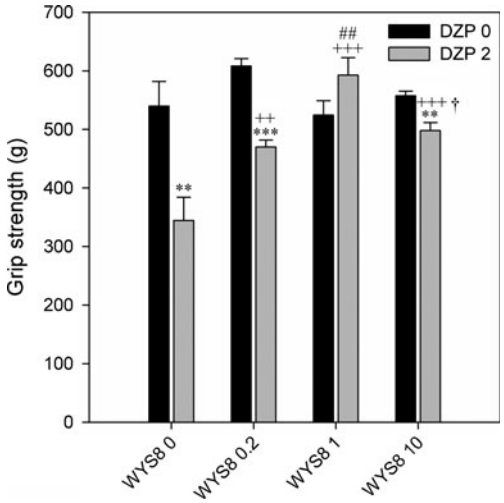

Fig. 6 The effects of WYS8 (0, 0.2, 1, and 10 mg/kg) in the absence (DZP 0) and the presence of diazepam 2 mg/kg (DZP 2), on the muscle tone measured in the grip strength test. Data points represent means± SEM. $^{**}p<0.01$ and $^{***}p<0.001$ compared with the DZP 0 group within the appropriate dose of WYS8; $^{++}p<0.01$ and $^{+++}p<0.001$ compared with the WYS8 0+DZP 2 group; $^{\#\#}p<0.01$ compared with the WYS8 0.2+DZP 2 group; $^{\dagger}p<0.05$ compared with the WYS8 1+DZP 2 group. Number of animals per treatment was 6, with the exception of WYS8 0.2 ($n=10$) and DZP 2+WYS8 10 group ($n=7$)

Rotarod

According to the two-way ANOVA, the effect of diazepam as a factor was highly significant ($F(2,80)=49.75$, $p<0.001$); the influence of WYS8 as well as diazepam× WYS8 interaction were insignificant ($F(3,80)=0.77$, $p=0.53$ and $F(6,91)=0.38$, $p=0.89$, respectively) (Fig. 7). Post hoc analysis has shown that 2 mg/kg diazepam significantly decreased rotarod performance in comparison to two other levels of diazepam (0 and 0.5 mg/kg) in the presence as well as in the absence of any dose of WYS8, showing that the incapacitating effect of diazepam in the rotarod test was not amenable to amelioration.

Pentylenetetrazole test

Chi-square test has shown that there was an overall difference in the frequency of incidence of clonic or tonic convulsions ($\chi^2=15.17$; $p=0.034$) (Fig. 8). Fisher exact test revealed that diazepam was effective in protecting the animals against convulsions induced by pentylenetetrazol ($p=0.019$). On the other hand, WYS8 on its own did not assure any reliable protection; moreover, there was a trend of dose-dependent deterioration in test performance (Fisher's exact test between 0.2 and 10 mg/kg doses of WYS8 was 0.093). The protective effect of diazepam disappeared when WYS8 was added, suggesting that full positive modulation at α_1 subunit-containing $GABA_A$ receptors was necessary for exerting the anticonvulsive effect of diazepam.

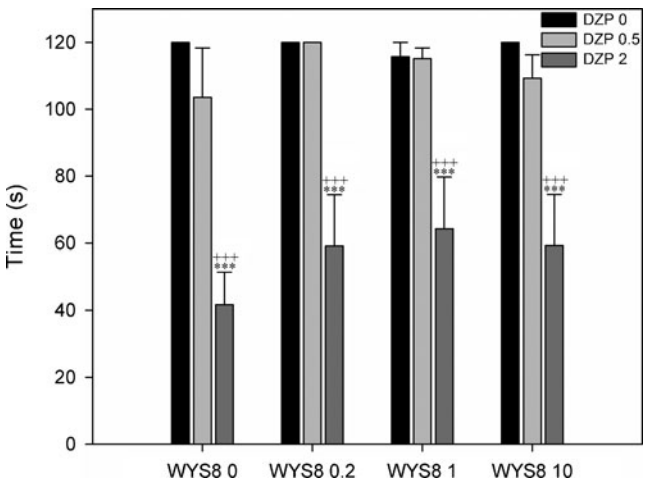

Fig. 7 The effects of diazepam (DZP; 0, 0.5, and 2 mg/kg) and WYS8 (0, 0.2, 1, and 10 mg/kg) on the time of rotarod performance. Data points represent means±SEM. ***$p<0.001$ compared with the DZP 0 group within the appropriate dose of WYS8; +++$p<0.001$ compared with the DZP 0.5 group within the appropriate dose of WYS8. Number of animals per treatment was 6–10, in total 92

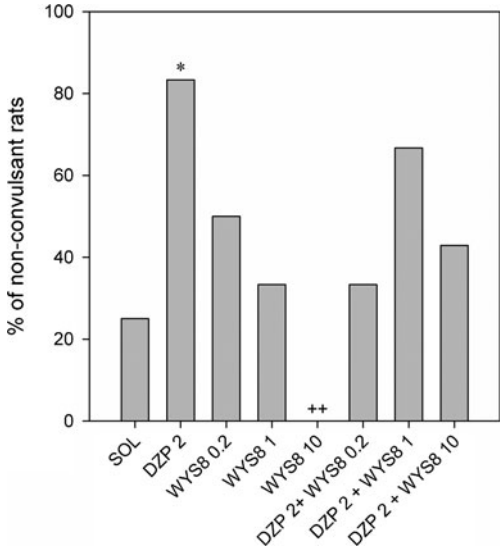

Fig. 8 The percent of non-convulsant (protected) animals treated with solvent (SOL), diazepam (DZP; 2 mg/kg), and WYS8 (0.2, 1, and 10 mg/kg), on their own and in combination, in the pentylenetetrazole test. Data points represent means. *$p<0.05$ compared with the SOL group; ++$p<0.01$ compared with the DZP 2 group. Number of animals per treatment was 6–12, in total 61

Discussion

The α_1 $GABA_A$ receptor population comprises more than 50 % of all $GABA_A$ receptors when receptors containing α_1 and another type of α subunit are added (Olsen and Sieghart 2008). Although elegant genetic studies with diazepam as a pharmacological tool revealed a crucial role of this major receptor class in the processes controlling sedation, motor coordination, and, in part, epileptic propensity (Rudolph and Möhler 2004), the results of these studies on their own are not sufficient to explain the complexity of findings obtained with novel ligands reportedly inactive at α_1 $GABA_A$ receptors (Skolnick 2012). In order to further elucidate this issue, it seems necessary to use pharmacologically selective modulation of effects of standard BZs at these receptors in wild-type animals. We have recently shown that WYS8, one of the most selective ligands for α_1 $GABA_A$ receptors reported to date (Yin et al. 2010), acts as a weak partial positive modulator at these receptors, devoid of behavioral activity in the water maze paradigm (Joksimović et al. 2013). Here, we report on the influence of WYS8 on diazepam-induced potentiation of recombinant $GABA_A$ receptors, and also on the further behavioral characterization in rats of WYS8 on its own and in combination with diazepam.

Twenty minutes after intraperitoneal injection, WYS8 at the dose of 10 mg/kg gave rise to a brain concentration of approximately 100 nM (Joksimović et al. 2013); at this concentration in vitro, WYS8 exerted weak potentiation of α_1 $GABA_A$ receptors, a borderline influence at α_2-, α_3-, and

α_4 $GABA_A$ receptors, and a lack of action at α_5- and $\alpha_6\beta_3\gamma_2$ $GABA_A$ receptors (present study). Such a pattern of activity could be related to the high affinity of WYS8 for $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors and low affinity for other, non-$\alpha_1\beta_3\gamma_2$ receptors (Yin et al. 2010 and unpublished results of BL Roth et al.). Moreover, the activity of WYS8 at rat recombinant $\alpha_1\beta_3\gamma_2$ receptors was not substantially different from that at β_1- or β_2-containing α_1 $GABA_A$ receptors, reproducing previous findings indicating that the subtype of β subunit does not significantly influence the effects of BZs at $GABA_A$ receptors (Hadingham et al. 1993).

Our unpublished data indicate that brain concentration of diazepam, measured 20 min after intraperitoneal administration of the 2 mg/kg dose, was approximately 1 μM, which is comparable to the concentration range reported in previous studies (Hironaka et al. 1984; Friedman et al. 1986). The present two-electrode voltage clamp results revealed that 100 nM WYS8 inhibited the diazepam-induced increase of the GABA current only at $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. A similar attenuating effect of WYS8 at these receptors was also observed when it was applied at tenfold higher (1 μM) but not at tenfold lower (10 nM) concentration, which indicates that the antagonizing effect of WYS8 was concentration-dependent. Taking all these in vitro results together, it appears that the efficacy profile of WYS8 reinforces its affinity data (Yin et al. 2010), indicating that it is one of the most selective ligands for α_1 $GABA_A$ receptors.

In addition to these lines of evidence, two caveats should be also addressed. Firstly, it is postulated that the channel-forming subunits expressed in cell lines cannot necessarily reproduce all properties of neuronal receptors (Birnir and Korpi, 2007), and, hence, native $GABA_A$ receptors may behave differently than recombinant receptors in an expression system (cf. Helms et al. 2012). This possibility, however, currently cannot be investigated in the absence of a clear and highly specific electrophysiological and pharmacological signature of the individual receptor subtypes that would allow their unequivocal identification in single-channel measurements from brain tissue. Secondly, synaptic $GABA_A$ receptors are expected to experience a brief high, rather than the low concentration of GABA, used in our study. As discussed in the methods section, "Experimental procedures," however, the effects of benzodiazepine site ligands at high GABA concentrations cannot be reliably measured due to the rapid desensitization of receptors that antagonizes the prolonged decay of GABA-induced currents elicited by such drugs under these conditions. Moreover, such a low GABA concentration corresponds well with that occurring at extrasynaptic receptors, which represent the majority of $GABA_A$ receptors in the brain (Kasugai et al. 2010), including substantial numbers of those containing the γ_2 subunit (Olsen and Sieghart 2008).

In the current behavioral characterization, diazepam, as a standard non-selective BZ, has expectedly engendered all the sedative, anxiolytic, muscle relaxant, ataxic, and anticonvulsant effects. On the other hand, WYS8 on its own was essentially devoid of BZ-like actions and only exhibited a hint of sedative-like potential at the highest tested dose of 10 mg/kg. This experimental finding suggests that a mild grade of sedation may be connected even with relatively small potentiation of α_1 $GABA_A$ receptors. Interestingly, a comparable degree of α_1 $GABA_A$ receptor activation induced by another partial positive modulator (MRK-409) has been discussed as possibly contributing to sedation in humans, an effect previously undetected in animal studies (Atack et al. 2011).

In regard to the activity-related parameters, it was evident in the spontaneous locomotor activity test that WYS8 may attenuate diazepam-induced locomotor depressant (i.e., sedative) effect, at least when overall activity was analyzed. Namely, there existed an interval (between 5 and 10 min after starting the test) when sedation was observable, despite the presence of WYS8. In the plus-maze, the activity-related parameters were changed in a different manner. In accordance with the enhanced aversiveness of the test conditions and the supposed higher baseline performance than in spontaneous locomotor test (Hogg 1996), diazepam on its own did not induce overt sedation, while addition of WYS8, especially at the highest dose, resulted in a significant depression of locomotion. Even though in vitro data showed that WYS8 significantly reduced diazepam's potentiation of α_1 $GABA_A$ receptors, it seems that administration of WYS8, depending on the behavioral task as well as time interval, could contribute to, deduct from, or leave unchanged the sedative action of diazepam in rats.

We have previously shown that selective antagonism at α_1 $GABA_A$ receptors by β-CCt may potentiate the anxiolytic effect of midazolam in the plus-maze (Savić et al. 2004). However, there were also opposite results showing that β-CCt was effective in antagonizing the anxiolytic-like influences of BZs (Belzung et al. 2000; Griebel et al. 1999). The present results revealed that a small dose (0.2 mg/kg) of the α_1-selective moderate positive modulator may unmask the anxiolytic action of the higher of the two tested doses of diazepam, possibly by reducing the overall activity at α_1 $GABA_A$ receptors through competition for the same binding sites and relative decrease of receptors occupied by the full positive modulator such as diazepam. This finding implies that the effects (most probably sedation) mediated by α_1 $GABA_A$ receptors may have hampered the anxiolytic actions of diazepam effected by other receptor subtypes. As a corollary, it cannot be excluded that a distinct level of activation of α_1 $GABA_A$ receptors may directly contribute to the anxiolytic-like action of BZs (cf. Lippa et al. 2005; Smith et al. 2012).

The study with β-CCt in monkeys (Licata et al. 2009) has indirectly supported the conclusion from knock-in mice that α_2 $GABA_A$, and, to a lesser degree, α_3 $GABA_A$ receptors are mainly involved in the muscle relaxation produced by BZs (Crestani et al. 2001). In the current grip strength test, WYS8, while having no effect per se, attenuated the myorelaxant effect of diazepam (2 mg/kg), especially at the 1 mg/kg dose; this finding was similar to the effect of β-CCt in the same test in rats (Milić et al. 2012). The results from the loaded grid test in mice have shown that β-CCt may significantly diminish the myorelaxant effect of diazepam administered at an intermediate dose of 3 mg/kg but not at doses of 1 or 10 mg/kg (Griebel et al. 1999). With these findings in mind, the present results suggest that muscle relaxation mediated by α_2 and α_3 $GABA_A$ receptors is somehow regulated by α_1 $GABA_A$ receptors, the particular manifestation of which depends on the relative level of activity at all three involved receptors.

The studies in monkeys (Licata et al. 2009; Platt et al. 2002) and knock-in mice (McKernan et al. 2000) have suggested that diazepam-induced loss of motor coordination is predominantly mediated by activation of α_1 $GABA_A$ receptors. Our recent results showed that the application of β-CCt fully antagonized the impairment of motor coordination induced by diazepam as well as zolpidem (Milić et al. 2012). The level of selective positive modulation at α_1 $GABA_A$ receptors obtained by WYS8 on its own was not sufficient to engender ataxia. However, the observed reduction of diazepam-induced activation at this receptor population, effected by WYS8 in vitro, was not sufficient even for attenuation of the diazepam's ataxic effect. These pieces of evidence suggest that some other, non-α_1 $GABA_A$ receptors may contribute to the ataxia, in settings when a moderate level of activation of α_1 $GABA_A$ receptors does exist.

The results from the pentylenetetrazole test revealed that the anticonvulsant effect of diazepam diminishes greatly when its activity at α_1 $GABA_A$ receptors is reduced by WYS8. Moreover, even a hint of proconvulsant action of 10 mg/kg WYS8 was noticed. In mice, it was shown that β-CCt did not affect the protective action of diazepam on pentylenetetrazole-induced convulsions (Griebel et al. 1999). Having in mind reports on the anticonvulsant activity of some ligands mainly inactive at α_1 $GABA_A$ receptors (Atack et al. 2006; Rivas et al. 2009), it appears that α_1 $GABA_A$ receptors may exert a kind of permissive action on the anti-convulsant activity of BZs mediated through non-α_1 $GABA_A$ receptors: The effect may be present when these receptors are potentiated fully or not at all, rather than in the case of a partial effect.

In conclusion, the partial activation at α_1 $GABA_A$ receptors effected by WYS8 may induce only negligible behavioral consequences. Its combination with diazepam was connected with a reduced sedation, muscle relaxation, and anticonvulsant activity, as compared with diazepam alone, whereas ataxia was preserved, while the anxiolytic effect of 2 mg/kg diazepam became unmasked. The anxiolytic and myorelaxant activity, although probably not attainable without positive modulation at the α_2/α_3-containing receptors, are prone to changes in both directions in dependence on the level of modulation at α_1 $GABA_A$ receptors. On the contrary, although it seems clear that anticonvulsant and even ataxic actions are attainable without substantial positive modulation at α_1 $GABA_A$ receptors, a strong activation of these receptors may be needed for the full-blown manifestation of these effects. Concerning efforts to develop novel BZ-like drugs, it appears that the relation between the activity at α_1 $GABA_A$ receptors and overall behavioral output is not simply "the less, the better", underlying the importance of a correct balance rather than individual actions at different $GABA_A$ receptor subtypes.

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Conflict of interest The authors have no competing interests with respect to this article.

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