The role of α_1 and α_5 subunit-containing $GABA_A$ receptors in motor impairment induced by benzodiazepines in rats

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Benzodiazepines negatively affect motor coordination and balance and produce myorelaxation. The aim of the present study was to examine the extent to which populations of γ-aminobutyric acid A ($GABA_A$) receptors containing α_1 and α_5 subunits contribute to these motor-impairing effects in rats. We used the nonselective agonist diazepam and the α_1-selective agonist zolpidem, as well as nonselective, α_1-subunit and α_5-subunit-selective antagonists flumazenil, βCCt, and XLi093, respectively. Ataxia and muscle relaxation were assessed by rotarod and grip strength tests performed 20 min after intraperitoneal treatment. Diazepam (2 mg/kg) induced significant ataxia and muscle relaxation, which were completely prevented by pretreatment with flumazenil (10 mg/kg) and βCCt (20 mg/kg). XLi093 antagonized the myorelaxant, but not the ataxic actions of diazepam. All three doses of zolpidem (1, 2, and 5 mg/kg) produced ataxia, but only the highest dose (5 mg/kg) significantly decreased the grip strength. These effects of zolpidem were reversed by βCCt at doses of 5 and 10 mg/kg, respectively. The present study demonstrates that α_1 $GABA_A$ receptors mediate ataxia and indirectly contribute to myorelaxation in rats, whereas α_5 $GABA_A$ receptors contribute significantly, although not dominantly, to muscle relaxation but not ataxia. *Behavioural Pharmacology* 23:191–197 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Benzodiazepines (BZs) were introduced into clinical practice in the beginning of the 1960s and, since then, have been widely prescribed as anxiolytic, hypnotic, anticonvulsant, and myorelaxant drugs. During the 1990s, it became clear that the pharmacological effects of BZs are mediated by positive modulation of four different subtypes of γ-aminobutyric acid A ($GABA_A$) receptors, namely those containing the α_1, α_2, α_3, or α_5 subunit, in addition to the γ_2 subunit (Sieghart, 2006). Genetic and pharmacological studies, by the means of the generation of mutant mouse lines [α_1(H101R), α_2(H101R), α_3(H126R), and α_5(H105R) knock-ins] (Rudolph and Möhler, 2004) and synthesis of novel, subtype-selective ligands, have helped in linking particular behavioral responses to specific $GABA_A$ receptor subtypes. The sedative effects of BZs were principally attributed to the α_1-$GABA_A$ receptor subtype, anxiolytic actions to α_2/α_3 containing receptors, anterograde amnesic effects to α_1/α_5 subtypes, and anticonvulsant activity partially to α_1-$GABA_A$ receptors (Löw *et al.*, 2000; McKernan *et al.*, 2000; Collinson *et al.*, 2002; Savić *et al.*, 2009).

BZs negatively affect motor coordination and balance, that is, they induce ataxia, which is, together with myorelaxation, often referred to as motor impairment (Verster *et al.*, 2002; Licata *et al.*, 2009). In contrast to ataxia, myorelaxation can be therapeutically desirable, and disentangling the molecular substrates of these two effects would benefit the development of compounds with an improved pharmacological profile. Like sedation, the impaired coordination and balance were also ascribed to potentiation at α_1-$GABA_A$ receptors and these results were consistent with experiments in both rodents and nonhuman primates (McKernan *et al.*, 2000; Platt *et al.*, 2002; Licata *et al.*, 2009). Ligands that lack or have substantially decreased activity at α_1-$GABA_A$ receptors, compared with conventional nonselective BZs, did not engender ataxia over the wide dose range tested (Licata *et al.*, 2005; Mirza *et al.*, 2008; Savić *et al.*, 2008; Atack 2010). The experiments on genetically modified mice have excluded the role of the α_1 subunit as a molecular substrate of myorelaxation (Rudolph *et al.*, 1999; McKernan *et al.*, 2000) and found that the myorelaxant properties of diazepam are mainly mediated by α_2-$GABA_A$ receptors; at very high doses of diazepam, the α_3-$GABA_A$ and α_5-$GABA_A$ receptor subtypes may also become implicated (Crestani *et al.*, 2001). However, a number of pharmacological studies have shown that muscle relaxation induced by nonselective BZ-site agonists could be reversed with the use of the α_1-$GABA_A$ selective antagonist β CCt, demonstrating ambiguity in this area (Griebel *et al.*, 1999; Licata *et al.*, 2009).

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The overall aim of the present study was to examine, by pharmacological means, the extent to which α_1-$GABA_A$ and α_5-$GABA_A$ receptor subtypes contribute to BZ-induced ataxia and muscle relaxation in Wistar rats and to provide further information on the molecular substrates of these two effects. Benzodiazepine-induced ataxia in rodents is usually measured using the rotarod test (Mirza *et al.*, 2008; Savić *et al.*, 2008), whereas the myorelaxant effects of BZs are often assessed using the grip strength test (Maurissen *et al.*, 2003). In the present study, we used diazepam, a ligand with high efficacy and no selectivity for $GABA_A$ receptor subtypes, and the α_1-$GABA_A$ receptor-selective agonist zolpidem, which possesses intermediate and no affinity for α_2/α_3-$GABA_A$ and α_5-$GABA_A$ receptor subtypes, respectively (Sanna *et al.*, 2002). Using the $GABA_A$ nonselective antagonist flumazenil, the α_1-subunit affinity-selective antagonist βCCt (Shannon *et al.*, 1984), and the α_5-subunit affinity-selective and efficacy-selective antagonist XLi093 (Li *et al.*, 2003), we examined the degree to which zolpidem-induced and diazepam-induced ataxia and myorelaxation could be antagonized.

Methods

Subjects

Male Wistar rats, weighing 200–230 g, were supplied by Military Farm, Belgrade, Serbia. Rats were housed in groups of six and were maintained under standard laboratory conditions (21 ± 2°C, relative humidity 40–45%) with free access to pellet food and tap water. They were kept on a 12 : 12 h light/dark cycle with lights on at 07.00 h. All handling and testing took place during the light phase of the diurnal cycle. Experiments were carried out in accordance with the European Economic Community Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Faculty of Pharmacy in Belgrade.

Rotarod test

Motor performance was assessed using an automated rotarod (Ugo Basile, Comerio, Italy). Before testing, rats were trained for 3 days until they could remain on a revolving rod for 120 s with acceleration from 15 rpm to 25 rpm. During the training days, all animals were given three training sessions of 2 min each, with a 30 min intersession interval. On the fourth day, rats that met the given criteria were selected for inclusion in the experiment. Groups of 6–8 animals received one of the following treatments: diazepam (0 and 2 mg/kg) in combination with βCCt (0, 1, 5, 20, and 30 mg/kg), flumazenil (0, 10, and 20 mg/kg), or XLi093 (0, 10, and 20 mg/kg), as well as zolpidem (0, 1, 2, and 5 mg/kg) and zolpidem (0 and 2 mg/kg) combined with βCCt (0, 5, and 20 mg/kg) or flumazenil (0, 10, and 20 mg/kg). Latency to falling off the rod was recorded automatically for each animal.

Grip strength test

This test was used to examine the myorelaxant properties of agonists, antagonists, and their combinations. Two experiments were performed: in the first, animals received diazepam (0 and 2 mg/kg) in combination with three levels of flumazenil (0, 10, and 20 mg/kg), βCCt (0, 20, and 30 mg/kg), and XLi093 (0, 10 and 20 mg/kg); in the second experiment, animals received zolpidem (0, 1, 2, and 5 mg/kg) and zolpidem (0 and 5 mg/kg) in combination with βCCt (0 and 10 mg/kg). After administration of the appropriate treatment, rats were allowed to grip, with their front paws, a metal trapezoid wire attached to a grip-strength meter (Ugo Basile, Italy). Grip strength was tested by dragging the rat gently by the tail. The apparatus measured the pull force (expressed in grams) necessary to overcome the animal's forelimbs grip-strength to the bar connected to a force transducer. Each animal was given three consecutive trials and the maximum value was taken.

Drugs

The compounds used were diazepam (Galenika, Belgrade, Serbia), zolpidem (Toronto Chemical Research, Toronto, Ontario, Canada), flumazenil (Feicheng BoYuan Fine Chemicals Co. Ltd, East Feicheng, China), XLi093 (4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid, 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-, 1,3-propanediyl ester), the α_5-subunit affinity-selective and efficacy-selective antagonist, and βCCt (*t*-butyl-β-carboline-3-carboxylate), the α_1-subunit affinity-selective antagonist; the latter two agents were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee, USA. The ligands were suspended in a solvent containing 85% distilled water, 14% propylene glycol, and 1% Tween-80. All animals received two intraperitoneal injections consisting of the appropriate ligand(s) and/or solvent (in a total volume of 2 ml/kg), 20 min before the testing. When a combination of two compounds was administered, the first compound was injected into the lower right and the second into the lower left quadrant of the peritoneum.

Statistics

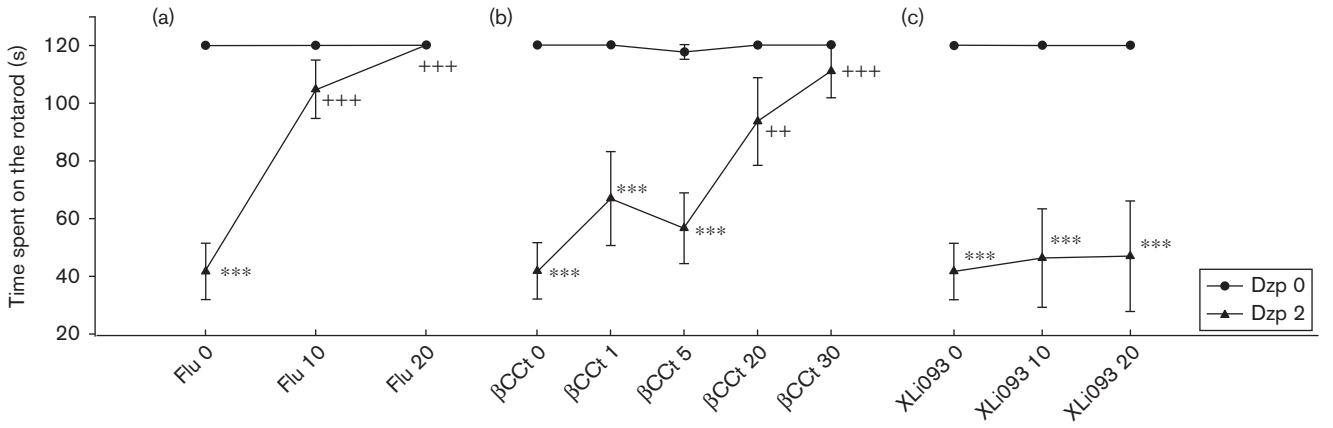
All numerical data presented in the figures are shown as the mean ± SEM. The dose response of zolpidem was assessed using one-way analysis of variance (ANOVA), with a post-hoc Student–Newman–Keuls test. The effects of combined treatments were assessed using two-way ANOVA with a post-hoc Student–Newman–Keuls test, where applicable.

Results

Rotarod

Animals that received 2 mg/kg diazepam spent significantly less time on the rotarod than the control group of rats (Fig. 1; $P < 0.001$). When diazepam was injected immediately after flumazenil, a significant main effect of

Fig. 1



The influence of pretreatment with antagonists (a) flumazenil (Flu), (b) βCCt, and (c) XLi093 on diazepam-induced (Dzp) ataxia on the rotarod. Data are mean ± SEM from $n = 8$ rats per group. ***P less than 0.001 versus vehicle; ^{++}P less than 0.01 versus 2 mg/kg diazepam; ^{+++}P less than 0.001 versus 2 mg/kg diazepam.

flumazenil [$F(2,40) = 18.07$, $P < 0.001$] and diazepam × flumazenil interaction [$F(2,45) = 18.07$, $P < 0.001$] were found. Both 10 and 20 mg/kg of flumazenil antagonized the motor incoordination induced by diazepam (Fig. 1a; both $P < 0.001$ compared with 2 mg/kg diazepam). Similarly, coadministration of βCCt resulted in a significant treatment effect [$F(4,68) = 4.05$, $P < 0.005$] and a significant diazepam × βCCt interaction [$F(4,77) = 3.83$, $P < 0.01$]. Although the two lower doses of βCCt (1 and 5 mg/kg) failed to antagonize the diazepam-induced motor impairment, coadministration of the two higher doses of βCCt (20 and 30 mg/kg) significantly increased the time spent on the rotarod (Fig. 1b; both $P < 0.001$), when compared with diazepam dosed at 2 mg/kg. XLi093, an α_5-selective antagonist, did not antagonize the diazepam-induced motor incoordination (Fig. 1c).

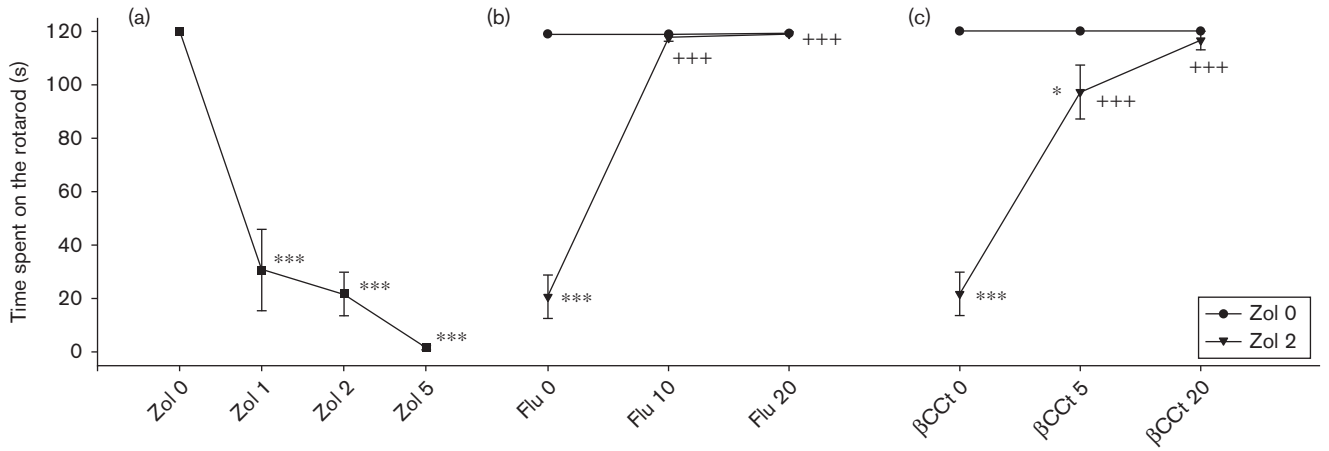
All three doses of zolpidem (1, 2, and 5 mg/kg) impaired motor coordination (Fig. 2a; $P < 0.001$ in all three cases). Pretreatment with flumazenil significantly influenced the zolpidem-induced ataxia [zolpidem: $F(1,37) = 114.02$, $P < 0.001$; zolpidem × flumazenil interaction: $F(2,42) = 108.54$, $P < 0.001$]. When compared with animals that received only 2 mg/kg of zolpidem, animals treated with the combination of zolpidem 2 mg/kg + flumazenil (10 or 20 mg/kg) spent significantly more time on the rotarod (Fig. 2b; $P < 0.001$ and $P < 0.001$, respectively). The effect on motor coordination of βCCt [$F(1,34) = 73.94$, $P < 0.001$] and the zolpidem × βCCt interaction [$F(2,39) = 40.61$, $P < 0.001$] was also significant. The subsequent post-hoc test showed that both 5 and 20 mg/kg of βCCt antagonized the zolpidem-induced ataxia (Fig. 2c; both $P < 0.001$, compared with 2 mg/kg zolpidem). There was also a significant difference in the time spent on the rotarod between animals that received 2 mg/kg zolpidem + 5 mg/kg βCCt and animals that received only 5 mg/kg βCCt ($P < 0.025$). None of the antagonists (flumazenil, βCCt, and XLi093) itself impaired the motor performance on the rotarod.

Grip strength

Application of 2 mg/kg diazepam produced significant muscle relaxation (Fig. 3; $P < 0.01$, relative to control). For the combination of diazepam + flumazenil, two-way ANOVA showed significant effects of diazepam [$F(1,31) = 6.09$, $P < 0.02$] and the flumazenil × diazepam interaction [$F(2,36) = 5.94$, $P < 0.01$); coadministration of flumazenil (10 and 20 mg/kg) reversed the diazepam-induced myorelaxation (Fig. 3a; $P < 0.001$ and $P < 0.01$, compared with diazepam 2 mg/kg, respectively). As with flumazenil, the effect of βCCt did not reach statistical significance, whereas the effect of diazepam [$F(1,28) = 7.82$, $P < 0.01$] as well as the interaction [$F(2,33) = 5.83$, $P < 0.01$] were significant. There were significant differences between the group that received 2 mg/kg diazepam and groups that received 2 mg/kg diazepam with either 20 or 30 mg/kg of ßCCt (Fig. 3b; $P < 0.05$ and $P < 0.001$, respectively). The assessment of the results obtained with the α_5-selective antagonist showed no significant effect of XLi093 on grip strength [$F(2,30) = 2.46$, NS], but showed a significant diazepam × XLi093 interaction [$F(2, 35) = 6.18$, $P < 0.01$]; the differences between groups that received diazepam + XLi093 (10 and 20 mg/kg) and the group that received diazepam were statistically significant (Fig. 3c; $P < 0.002$ and $P < 0.005$, respectively).

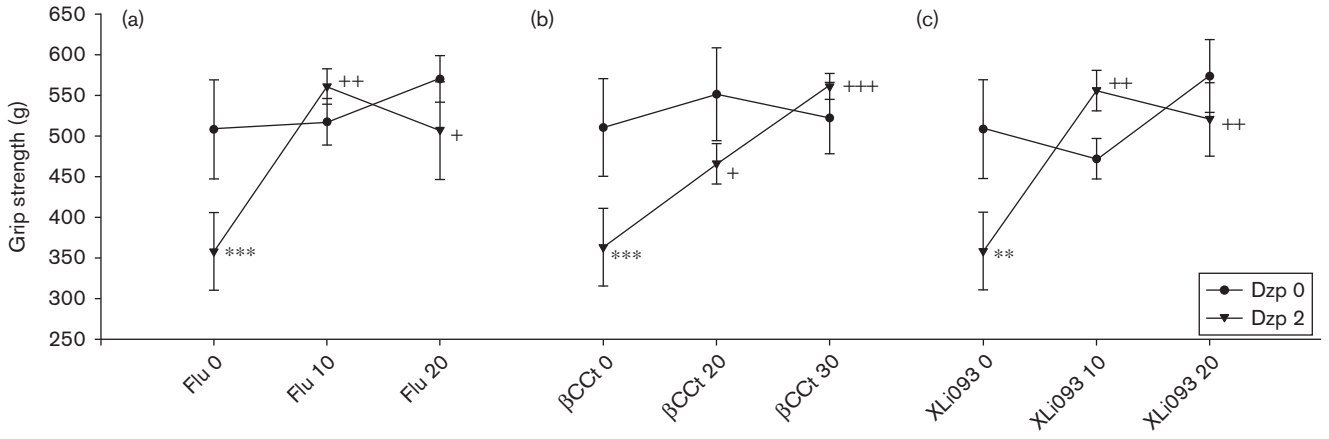
Zolpidem significantly decreased grip strength [$F(3,20) = 10.34$, $P < 0.001$]. Muscle relaxation was significant with 5 mg/kg zolpidem ($P < 0.001$), whereas the two lower doses (1 and 2 mg/kg) were at the control level (Fig. 4a). When the combination 5 mg/kg zolpidem + 10 mg/kg ßCCt was assessed, significant effects of

Fig. 2



(a) Effects of zolpidem (Zol) on rotarod performance and the influence of pretreatment with (b) flumazenil (Flu) and (c) βCCt on ataxia induced by zolpidem (2 mg/kg). Data are mean ± SEM from $n = 6$ rats per group. *P less than 0.05 versus vehicle; ***P less than 0.001 versus vehicle; ^{+++}P less than 0.001 versus 2 mg/kg zolpidem.

Fig. 3



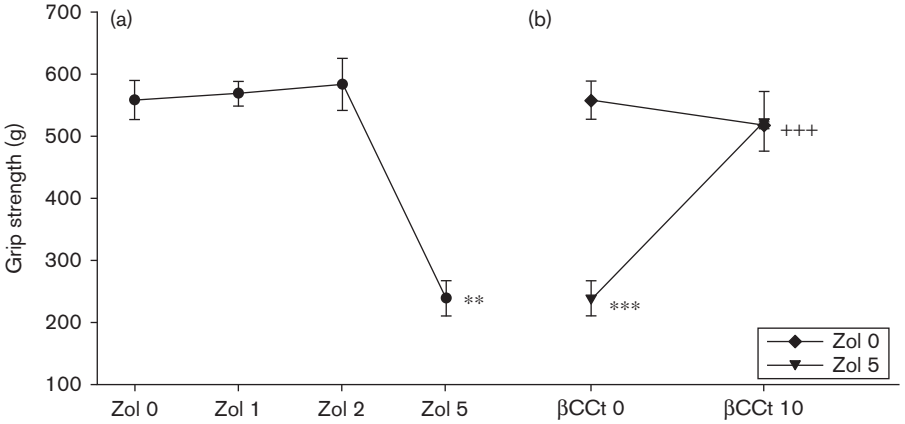
The influence of pretreatment with the antagonists (a) flumazenil (Flu), (b) βCCt, and (c) XLi093 on the diazepam-induced (Dzp) muscle relaxation measured in the grip strength test. Data are mean ± SEM from $n = 8$ rats per group. **P less than 0.05 versus vehicle; ***P less than 0.001 versus vehicle; ^{+}P less than 0.05 versus 2 mg/kg diazepam; ^{++}P less than 0.01 versus 2 mg/kg diazepam; ^{+++}P less than 0.001 versus 2 mg/kg diazepam.

zolpidem [$F(1,15) = 19.74$, $P < 0.001$], βCCt $F(1,15) = 16.11$, $P < 0.001$] and their interaction [$F(1,18) = 27.53$, $P < 0.001$] were found. Although βCCt itself did not alter grip strength, its addition to zolpidem reversed the zolpidem-induced muscle relaxation (Fig. 4b; $P < 0.001$, compared with 5 mg/kg zolpidem).

Discussion

Studies on genetically modified mice, in which a distinct α subunit of $GABA_A$ receptors is rendered insensitive to diazepam, represent valuable tools in revealing which receptor subtype is necessary for the expression of a specific behavioral response. These experiments pointed toward α_1-$GABA_A$ receptors as the main subtype in eliciting ataxia in mice (McKernan *et al.*, 2000). In the present study, diazepam-induced and zolpidem-induced ataxia on the rotarod in rats was successfully antagonized with the α_1-selective antagonist βCCt. Because of its 20-fold selectivity for α_1-$GABA_A$ receptors compared with α_2-$GABA_A$ and α_3-$GABA_A$ receptors, βCCt is one of the most selective BZ-site ligands identified to date (Cox *et al.*, 1995; Huang *et al.*, 2000). In many behavioral studies, βCCt successfully reversed the effects of BZs related to the α_1-$GABA_A$ receptor subtype, such as ataxia, sedation, and anticonvulsant activity (Griebel *et al.*, 1999; Platt *et al.*, 2002; Savić *et al.*, 2009). However, not

Fig. 4



(a) Muscle-relaxant effect of zolpidem (Zol) and (b) the influence of pretreatment with βCCt. Data are mean ± SEM from $n = 6$ rats per group. $^{**}P$ less than 0.05 versus vehicle; $^{***}P$ less than 0.001 versus vehicle; ^{+++}P less than 0.001 versus 5 mg/kg zolpidem.

all experiments using βCCt as the α_1-selective ligand have reported antagonism of the diazepam-induced ataxia in mice or rats. Such discrepancies may have resulted from differences in the experimental design. Shannon and colleagues, (1984) reported that administration of 30 mg/kg βCCt did not attenuate the diazepam-induced ataxia in mice. The degree of motor impairment was assessed using an inverted-screen test, where the concomitant myorelaxation was likely to influence the performance of the test. Another study found that motor incoordination engendered by diazepam, triazolam, and zolpidem in mouse pups was not sensitive to βCCt (Rowlett *et al.*, 2001). However, motor impairment was related to rolling motions, as opposed to normal locomotor activity of mouse pups, and probably involved a predominantly spinal mechanism and engagement of α_2-$GABA_A$ and α_3-$GABA_A$ receptor subtypes (McKernan and Whiting, 1996). In the present study, the dose of βCCt needed to antagonize zolpidem-induced ataxia was substantially lower than the dose that antagonized the effect of diazepam (5 vs. 20 mg/kg). This implies that an effect of diazepam, possibly myorelaxation, mediated by receptors other than the α_1-$GABA_A$ receptor, may have contributed to the influence of diazepam, but not zolpidem, on rotarod test performance. In this scenario, the dose of 20 mg/kg of βCCt may have either blocked the α_1-$GABA_A$ receptor population more completely or started to prevent binding of diazepam to non-α_1-$GABA_A$ receptors.

The possibility that the α_5-$GABA_A$ receptor subtype plays a modulatory role in behavioral effects predominantly conferred by the α_1 subunit, such as sedation, tolerance development, and memory impairment, has been previously proposed (van Rijnsoever *et al.*, 2004; Savić *et al.*, 2008; Savić *et al.*, 2009). Hence, we tested the ability of the α_5 selective antagonist XLi093 to influence the diazepam-induced ataxia. At the dose of 20 mg/kg, which was previously shown to intensify diazepam-induced sedation (Savić *et al.*, 2009), XLi093 did not significantly affect the motor-impairing effect of diazepam. This means that ataxia, as assessed in the rotarod test in rats, is not dependent on the activation of α_5-$GABA_A$ receptors.

Although genetic studies did not detect any role of the α_1 subunit in mediating muscle relaxation (Rudolph *et al.*, 1999; McKernan *et al.*, 2000), the data from experiments with subtype-selective ligands varied from one study to another depending on the species used and the dose of agonist or antagonist applied (Griebel *et al.*, 1999; Elliot and White, 2001; Licata *et al.*, 2009). In a radiotelemetric study in rats, zolpidem at the dose of 5 mg/kg, but not 2.5 mg/kg, induced a significant decrease in electromyographic activity, a parameter aimed to assess muscle relaxation (Elliot and White, 2001). In the present study, significant myorelaxation observed after both diazepam and zolpidem administration was prevented by pretreatment with βCCt. As the dose of zolpidem producing myorelaxation (5 mg/kg) was substantially higher than the minimal dose that induced ataxia (1 mg/kg), the possibility that zolpidem-induced myorelaxation is not mediated by α_1-$GABA_A$ receptors needs to be discussed. Despite its binding preference for α_1-$GABA_A$ receptors, zolpidem also binds to and potentiates effects at α_2-$GABA_A$ and α_3-$GABA_A$ receptors (Sanna *et al.*, 2002). The in-vivo selectivity of zolpidem for the α_1-enriched cerebellum, in contrast to α_2/α_3-enriched spinal cord, assessed through the reduction in flumazenil binding, is generally less than the α_1 selectivity of this compound *in vitro* (Atack *et al.*, 1999). However, the displacement curve for zolpidem in the spinal cord of rats (Benavides *et al.*, 1992) and mice (Atack *et al.*, 1999) is relatively flat, and very high doses of zolpidem (> 30 mg/kg in mice; Atack *et al.*, 1999) are

needed for half-inhibition of radio-labeled flumazenil binding in this region predominantly implicated in GABA-mediated myorelaxation (Bohlhalter *et al.*, 1996). Thus, one can conclude that muscle-relaxant effect of zolpidem at the dose of 5 mg/kg may not be exclusively mediated by α_2-$GABA_A$ receptors, the subtype largely responsible for the muscle-relaxant effect of diazepam (Crestani *et al.*, 2001). In contrast, βCCt (30 mg/kg) reversed diazepam-induced muscle relaxation in mice (Griebel *et al.*, 1999), and at the dose of 3 mg/kg, it attenuated the myorelaxant properties of several nonselective benzodiazepine agonists in squirrel monkeys (Licata *et al.*, 2009). The propensity of βCCt to antagonize some of the principally non-α_1-mediated effects of diazepam was also shown in the elevated plus-maze and light-dark test of anxiety (Griebel *et al.*, 1999; Belzung *et al.*, 2000). Nonetheless, a potentiating effect of 30 mg/kg βCCt on the anxiolytic actions of BZs in rats has also been repeatedly reported (Savić *et al.*, 2004, 2005), which cannot be a consequence of putative antagonism on α_2-$GABA_A$ receptors. Assessment of the ability of 10 mg/kg βCCt (intraperitoneally) to displace the radio-labeled flumazenil in mice indicates that βCCt at the given dose level preferentially targets the cerebellum, whereas it binds to less than 40% of $GABA_A$ receptors, mainly of the α_2-subtype, in the spinal cord (Rowlett *et al.*, 2005). Given the doses of zolpidem and βCCt that we used, we hypothesize that under our experimental conditions, the actions of these ligands may, to a small extent, have involved the α_2-$GABA_A$ receptor subtype, in addition to the predominantly affected α_1-$GABA_A$ receptor subtype. In the presence of intense activation of α_1-$GABA_A$ receptors by a large dose of zolpidem, the presumed small involvement of α_2-$GABA_A$ receptors may have been large enough to trigger muscle relaxation.

The contribution of the α_5 subunit in mediating the muscle-relaxant effect of diazepam was observed in α_5 (H105R) mutant mice (Crestani *et al.*, 2002). Here, we report on antagonism of the muscle-relaxant effect of diazepam with the α_5-selective ligand XLi093 in rats. Nonetheless, muscle relaxation can be achieved without the apparent activation of α_5-$GABA_A$ receptors, as demonstrated in experiments with zolpidem (Elliot and White, 2001; Licata *et al.*, 2009). Furthermore, an α_2/α_3 selective compound devoid of agonistic activity at the α_5 subunit exerted muscle relaxation in monkeys (Licata *et al.*, 2005). These results suggest that the role of the α_5 subunit in the BZ-induced myorelaxation could be described as nondominant, but still significant, and should be further investigated.

The present study demonstrates that α_1-$GABA_A$ and α_5-$GABA_A$ receptor subtypes differentially contribute to the motor-impairing effects of BZs in rats. Although activation of α_1-$GABA_A$ receptors is a prerequisite for eliciting ataxia, these receptors are probably not directly involved in mediating muscle relaxation but still may contribute to the manifestation of this effect triggered by a small fraction of activated α_2-$GABA_A$ receptors. In contrast, activation of α_5-$GABA_A$ receptors contributes significantly, although not dominantly, to muscle relaxation, but not ataxia. Thus, in the quest for ligands with an improved pharmacological profile, it could be of importance to avoid substantial potentiation through α_1 subunits, if ataxia is to be prevented, whereas a certain level of activation at both α_1 and α_5 subunits could be advantageous when muscle relaxation is required.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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