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The effects of zolpidem treatment on $GABA_A$ receptors in cultured cerebellar granule cells: Changes in functional coupling

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ABSTRACT

Aims: Hypnotic zolpidem is a positive allosteric modulator of γ-aminobutyric acid (GABA) action, with preferential although not exclusive binding for α1 subunit-containing $GABA_A$ receptors. The pharmacological profile of this drug is different from that of classical benzodiazepines, although it acts through benzodiazepine binding sites at $GABA_A$ receptors. The aim of this study was to further explore the molecular mechanisms of $GABA_A$ receptor induction by zolpidem.

Main methods: In the present study, we explored the effects of two-day zolpidem (10 μM) treatment on $GABA_A$ receptors on the membranes of rat cerebellar granule cells (CGCs) using [^{3}H]flunitrazepam binding and semi-quantitative PCR analysis.

Key findings: Two-day zolpidem treatment of CGCs did not significantly affect the maximum number (B_{max}) of [^{3}H]flunitrazepam binding sites or the expression of α1 subunit mRNA. However, as shown by decreased GABA [^{3}H]flunitrazepam binding, two-day exposure of CGCs to zolpidem caused functional uncoupling of GABA and benzodiazepine binding sites at $GABA_A$ receptor complexes.

Significance: If functional uncoupling of GABA and benzodiazepine binding sites at $GABA_A$ receptors is the mechanism responsible for the development of tolerance following long-term administration of classical benzodiazepines, chronic zolpidem treatment may induce tolerance.

Introduction

Gamma-aminobutyric acid type A ($GABA_A$) receptors are pentameric complexes of subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π) that form central anion channels that mediate inhibition in the central nervous system (Korpi et al., 2002). The α1 subunit receptors are the most abundant subtype of synaptic $GABA_A$ receptors expressed in the brain (Olsen and Sieghart, 2008). $GABA_A$ receptors can be modulated by a variety of pharmacologically and clinically relevant drugs, such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics, etc.

Zolpidem, an imidazopyridine with a chemical structure different from that of classical benzodiazepines, exhibits high selectivity for α1 subunit-containing $GABA_A$ receptors (Depoortere et al., 1986; Olsen and Sieghart, 2008). Using a knock-in approach, Crestani et al. (2000) confirmed the preferential affinity of zolpidem for the α1 subtype of $GABA_A$ receptors, and Cope et al. (2004) demonstrated the contribution of the γ2 subunit to its action in vivo. In addition to its pronounced sedative properties and mild anxiolytic and myorelaxant effects (Depoortere et al., 1986; Sanger et al., 1996), several published papers have suggested that zolpidem might have better anticonvulsant activity than previously thought (Crestani et al., 2000; Peričić et al., 2008; Vlainić and Peričić, 2010).

Long-term administration of positive allosteric modulators of $GABA_A$ receptors, such as benzodiazepines, often results in the development of tolerance and dependence, phenomena accompanied by different adaptive changes in the GABAergic system. These changes mainly lead to altered receptor expression and/or function. While the observed changes in receptor expression have been inconsistent (Wafford, 2005), many researchers working either on animals (Gallager et al., 1984), neuronal cultures (Roca et al., 1990; Friedman et al., 1996) or recombinant receptors (Primus et al., 1996; Ali and Olsen, 2001; Peričić et al., 2007; Švob Štrac et al., 2008; Vlainić et al., 2010) have found functional uncoupling of allosteric linkages between GABA and benzodiazepine binding sites, characterized by decreased ability of benzodiazepines to potentiate the action of GABA as well as by decreased ability of GABA to enhance benzodiazepine binding (Gallager et al., 1984). It has been suggested that this phenomenon may be related to the development of tolerance to benzodiazepine action. However, despite many studies on this topic, the molecular mechanisms involved in the development of tolerance and dependence following long-term treatment with benzodiazepines are still unclear (Bateson, 2002; Wafford, 2005; Uusi-Oukari and Korpi, 2010).

The first studies in rodents suggested that, unlike benzodiazepines that bind non-selectively, repeated treatment with zolpidem does not

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result in tolerance and dependence (Perrault et al., 1992). In contrast, our previous study demonstrated development of anticonvulsant and sedative tolerance after repeated (twice daily for 10 days) zolpidem treatment in mice, similar to that produced by prolonged diazepam treatment (Vlainić and Peričić, 2009).Moreover, the results of another study (Vlainić et al., 2010) suggested that two-day zolpidem treatment of human embryonic kidney (HEK) 293 cells stably expressing recombinant $\alpha1\beta2\gamma2s$ $GABA_A$ receptors up-regulated the number of [^{3}H]flunitrazepam, [^{3}H]muscimol and [^{3}H]TBOB (t-butylbicycloorthobenzoate, a radioligand for the channel site) binding sites and impairs allosteric interactions between GABA and benzodiazepine binding sites. To elucidate further the molecular mechanisms of zolpidem treatment, we applied the same two-day protocol on cultured rat cerebellar granule cells (CGCs) in the present study.

CGCs constitute the majority of neurons in the cerebellum and express 14 different subunits ($\alpha1$-6, $\beta1$-3, $\gamma1$-3 and δ) of $GABA_A$ receptors (Bovolin et al., 1992), but with a different expression patterns than that observed in the cerebellum of adult rats (Laurie et al., 1992). Radioligand binding studies and semi-quantitative RT-PCR were used to explore the effects of zolpidem treatment on the expression and functional coupling of $GABA_A$ receptors expressed on the membranes of CGCs.

Materials and methods

Materials

The chemicals that were used in the study were purchased from a variety of suppliers. Culture medium, antibiotics and fetal bovine serum were supplied from Invitrogen/Gibco (Grand Island, NY, USA). Trypsin, deoxyribonuclease (DNase), poly-L-lysine, cytosine arabinofuranoside, GABA and diazepam were purchased from Sigma. Zolpidem tartrate (N,N,6-trimethyl-2-(4-methylphenyl)-imidazo-(1,2-a)pyridine-3-acetamide) was generously donated by its manufacturer (Pliva, Zagreb, Croatia). [^{3}H]Flunitrazepam (specific activity 87 Ci/mmol) was purchased from Amersham Biosciences UK Ltd.

Cell culture

Primary cultures of granule cells were isolated from the cerebellum of rats on postnatal day 8 according to the procedure adapted from Oberdoerster and Rabin (1999). Briefly, the cerebella from decapitated rats were dissected out and minced, and then, the tissue was digested at 37 °C for 15 min with 0.125% trypsin and 0.2% DNase. The trypsin was inactivated by the addition of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. The cell pellet was centrifuged at $1000\times g$ for 5 min, re-suspended in DMEM, filtered through a 100-μm nylon mesh and centrifuged again. The remaining cell pellet was re-suspended in DMEM. The cells were plated (2×10^6 cells per cm^2) in a dish that was coated with poly-L-lysine (10 μg/ml, Mr = 70,000–150,000) and maintained at 37 °C in a 5% CO_2 atmosphere. The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM KCl (a high concentration of K^+ is necessary to induce membrane depolarization to maintain viable granule cells in culture) and 50 mM glucose. Anti-mitotic cytosine arabinofuranoside (AraC; final concentration 10 μM) was added to cultures on day 1 after isolation to inhibit proliferation of non-neuronal cells. The culture medium was replaced every day with fresh medium. The cells were maintained in culture for a total of 8 days, and two-day treatment with zolpidem (dissolved in distilled water and diluted in culture medium to a final concentration of 10 μM) was initiated accordingly.

The cells were used in experiments following culturing in vitro for 8 days to allow cell maturation and stabilization of adult channel conductance patterns. At that time, cerebellar cultures contained more than 95% granule cells and less than 5% glia cells. In addition, the granule cells express functional $GABA_A$ receptors (Bovolin et al., 1992) with a subunit composition similar to that of the cerebellum during postnatal development, but with a different pattern than that for the cerebellum of adult rats in which only several α subunits are expressed (Laurie et al., 1992).

All experimental procedures in the present study were approved by the Ruđer Bošković Institute's Animal Research Committee and were performed in accordance with the Principles of National Institute for the Care and Use of Laboratory Animals (NIH publication No. 86–23, revised 1996) and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. All efforts were made to minimize animal suffering and to reduce the number of animals used.

[^{3}H]flunitrazepam binding assay

Membranes from cultured CGCs were prepared with modifications of the method described previously (Peričić et al., 2004). The cells were washed with phosphate-buffer saline (PBS), scraped from flasks into ice-cold PBS and centrifuged at $12{,}000\times g$ for 12 min. The cell pellet was homogenized in 50 mM Tris-citrate buffer, pH 7.4, using a teflon pestle and a glass homogenizer and then centrifuged at $200{,}000\times g$ for 20 min. The same procedure was repeated two more times (re-suspension/centrifugation). Finally, the pellet was re-suspended and stored in aliquots at −20 °C. The suspension of the cell membranes was centrifuged on the day of [^{3}H]flunitrazepam binding assay once more at $200{,}000\times g$ for 20 min.

In vitro benzodiazepine binding was assays were performed on membrane preparations, as previously described, using [^{3}H]flunitrazepam as a radioligand (Peričić et al., 2005). Aliquots of cell membrane preparations were incubated in 50 mM Tris-citrate buffer supplemented with 150 mM NaCl at 4 °C for 90 min with a fixed concentration (1 nM) of [^{3}H] flunitrazepam. In stimulation studies (GABA-shift), GABA (final concentration 100 μM) was added and incubated with [^{3}H]flunitrazepam. Non-specific binding was determined by [^{3}H]flunitrazepam bound to the cell membranes in the presence of 100 μM diazepam and represented less than 20% of total binding. The total assay volume of all binding studies was 0.5 ml. After rapid vacuum filtration, filters (Whatman GF/C) were dried overnight and then soaked in a liquid scintillation cocktail. Bound radioactivity was quantified by liquid scintillation counting on a β-scintillation counter (Perkin Elmer, Wallace 1409DSA).

Protein concentration assays

Membrane protein concentrations were determined in 10 μL of membrane suspension using bovine serum albumin as a standard.

Semi-quantitative RT-PCR

Total cellular RNA was extracted using a High Pure RNA Isolation Kit (Roche) and quantified at 260 nm using a spectrophotometer. Reverse transcription and semi-quantitative PCR were performed in a PerkinElmer 9600 thermocycler, as previously described (Jazvinšćak Jembrek et al., 2008). Together with random hexadeoxynucleotide primers (2.5 μM), total RNA (1 μg) was denatured at 65 °C for 5 min, and the first strand of cDNA was synthesized by adding the following reagents: reverse transcription buffer (Invitrogen), 0.5 mM dNTPs (Roche), 40 U of RNase-inhibitor (Roche) and 200 U of SuperScript II reverse transcriptase (Invitrogen). For DNA synthesis, after primer annealing (25 °C, 10 min), the reaction mixture was incubated at 42 °C for 50 min and then heated (70 °C, 15 min) for enzyme inactivation. Each RT reaction had two negative controls: the sample without SuperScript II reverse transcriptase and the sample without the

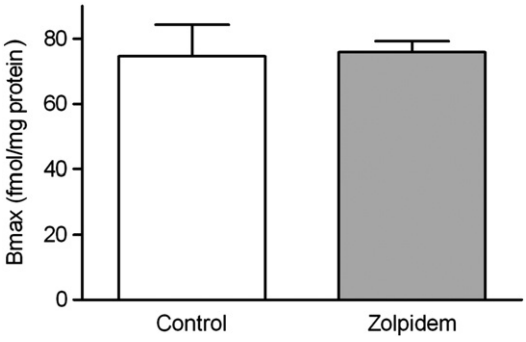

Fig. 1. The effect of two-day zolpidem treatment on the expression of [^{3}H]flunitrazepam binding sites on membranes obtained from CGCs. The results are expressed as the mean ± SEM (n = 3).

RNA template to test for contamination with genomic DNA. Expression of the housekeeping gene β-actin was used as an internal standard. The resulting cDNA (1:5 dilution) was amplified by PCR with 1.25 U of Taq DNA polymerase (Promega) in 20 μL of standard buffer containing 0.2 μM of each sense and antisense primers (Invitrogen), 1.5 mM $MgCl_2$ and 200 μM of dNTP mix. PCR primers used in this study were as follows: β-actin, TCA CCA ACT GGG ACG ACA TG and TTC GTG GAT GCC ACA GGA CT; $GABA_A$ receptor α1 subunit, AGC TAT ACC CCT AAC TTA GCC AGG and AGA AAG CGA TTC TCA GTG GAG AGG. Cycle parameters included an initial denaturation step (95 °C, 5 min), followed by denaturation (95 °C, 30 s), annealing (60 °C, 30 s) and extension (72 °C, 1 min). Preliminary experiments were performed with each set of primers to assess linearity of PCR product amplification with respect to cycle number. The PCR products of both the α1 subunit and β-actin gene were measured during the log phase of the reaction (35 cycles). Samples of the reaction products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Maximal optical density was obtained with the Image Master VDS software (Pharmacia). The expression of α1 subunit mRNA was normalized to β-actin mRNA expression and compared between the control and zolpidem-treated groups.

Data analysis

The analysis of binding data for [^{3}H]flunitrazepam binding was performed using a method for calculating receptor number (B_{max}) from competitive binding data (DeBlasi et al., 1989) as follows:

$$B_0 = dpm_{TOTAL} - dpm_{NONSPECIFIC}/c \times 2220 \times \text{mg protein}$$
$$B_{max} = B_0 \times IC_{50}/L$$

where dpm is the disintegrations per minute, c is the concentration of radioactive ligand, 2220 is the number of disintegrations of 1 nCi per minute, IC_{50} (nM) is the affinity of drug for receptor and L (nM) is the concentration of radioactive ligand in the assay. These equations are based on several assumptions: labeled and unlabeled ligand have identical affinities for the receptor, only one class of binding site exists, there is no cooperation between binding sites and only a small fraction of the total ligand is bound to receptors.

The percentage of change in [^{3}H]flunitrazepam binding produced by the presence of GABA was defined as (specific binding in the presence of GABA/specific binding in the absence of GABA) × 100.

The results are presented as the means ± SEM of at least three independent experiments. The means ± SEM for maximum enhancement (E_{max}) of [^{3}H]flunitrazepam binding were determined from several individual experiments performed in duplicate.

Statistical analyses of the results were conducted by Student's *t*-test. P-values less than 0.05 were considered significant.

Results

The effect of two-day zolpidem treatment on [^{3}H]flunitrazepam binding to membranes isolated from cultured CGGs

Cultured rat CGCs were treated for two days with 10 μM zolpidem. Cell membranes were prepared as described in the Materials and methods and incubated in the presence of 1 nM [^{3}H]flunitrazepam. The maximum number (B_{max}) of [^{3}H]flunitrazepam binding sites were calculated using the formula for calculating receptor number from binding data (DeBlasi et al., 1989). The B_{max} values were 74.62 ± 9.67 fmol/mg protein in the control group and 75.88 ± 3.38 fmol/mg protein in the zolpidem-treated group (n = 3). As shown in Fig. 1, there was no significant difference between the maximum number (B_{max}) of benzodiazepine binding sites on the membranes of cells obtained from control and zolpidem-treated cells.

The effect of two-day zolpidem treatment on $GABA_A$ receptor expression

Isolated rat CGCs were treated for two days with 10 μM zolpidem, and the level of $GABA_A$ receptor α1 subunit mRNA was determined by semi-quantitative RT-PCR analysis. The maximal optical density of the α1 subunit band was normalized to the expression of the housekeeping gene β-actin. Incubation of CGCs in the presence of zolpidem had no significant effect on the abundance of $GABA_A$ receptor α1 subunit mRNA relative to control cultures (Fig. 2).

The effect of two-day zolpidem treatment on allosteric coupling between GABA and benzodiazepine binding sites at $GABA_A$ receptor complexes

The effect of zolpidem treatment on GABA potentiation of [^{3}H] flunitrazepam binding as a measure of degree of allosteric linkage between GABA and benzodiazepine binding sites at $GABA_A$ receptor complexes was studied with membranes of cerebellar granule neurons incubated for two days in the presence of 10 μM zolpidem. The GABA-shift assay was performed in the presence of 100 μM GABA

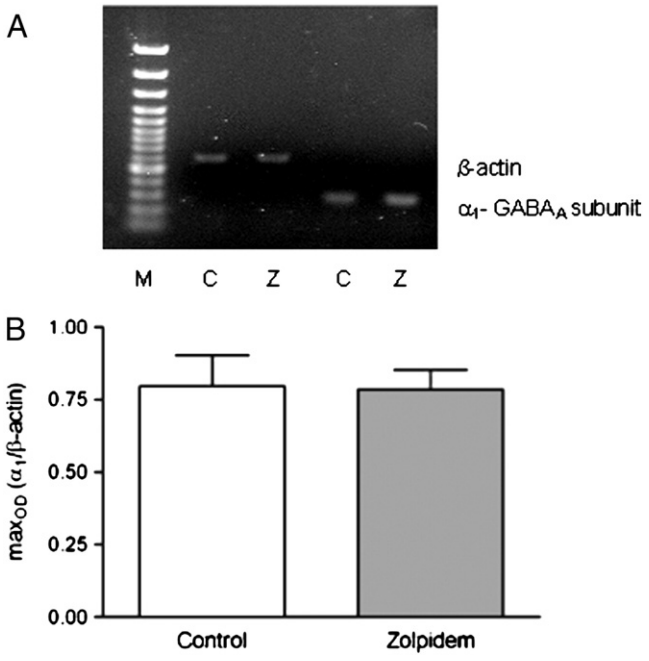

Fig. 2. The effect of two-day zolpidem treatment on the expression of $GABA_A$ receptor α1 subunit mRNA (A) Representative agarose gel electrophoresis; (B) Results of RT-PCR analysis. Abbreviations: size marker (M), control cells (C) zolpidem-treated cells (Z). Data are expressed as the means ± SEM from six independent RT-PCR analyses (with PCR performed in triplicate) after three separate preparations of total RNA.

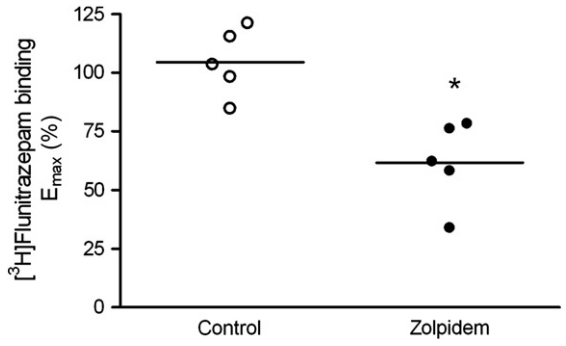

Fig. 3. The effect of two-day zolpidem treatment on GABA potentiation of [^{3}H] flunitrazepam binding to membranes from rat CGCs. Data are expressed as the percentages of basal values. The bars are means ± SEM (n = 5). *P<0.003 vs. control (Student's *t*-test).

with 1 nM radioactive ligand. The maximum enhancement (E_{max}) of [^{3}H]flunitrazepam binding produced by GABA in the control group was 104.50 ± 6.44%, indicating that the GABA binding site was functionally coupled to the benzodiazepine binding site. In the zolpidem-treated group, the E_{max} values were significantly lower (61.66 ± 7.95%). As shown in Fig. 3, the results obtained indicated that two-day zolpidem treatment decreased the level of allosteric coupling between GABA and benzodiazepine binding sites at $GABA_A$ receptor complexes (P<0.003).

Discussion

Zolpidem is a positive allosteric modulator of GABA action with selectivity for α1 subunit-containing $GABA_A$ receptors. It was previously thought that zolpidem causes fewer side effects and a lower degree of tolerance during long-term administration than benzodiazepines (Perrault et al., 1992; Sanger et al., 1996). However, mice treated repeatedly with zolpidem, similar to those treated repeatedly with diazepam, have been found to develop tolerance to its sedative and anticonvulsant effects (Vlainić and Peričić, 2009). Similar results were obtained in rats (Auta et al., 2008). In addition, the World Health Organization reports that the frequency of zolpidem abuse and dependence is similar to that of benzodiazepines. Moreover, it has been emphasized that there are not enough studies on the use of high doses of zolpidem.

To extend our previous work suggesting that two-day zolpidem treatment up-regulates the expression of α1β2γ2s $GABA_A$ receptors in HEK 293 cells (Vlainić et al., 2010), we investigated the effect of two-day zolpidem (10 μM) treatment on $GABA_A$ receptors expressed in primary rat CGCs. The results demonstrated that treatment with zolpidem failed to change the maximum number (B_{max}) of [^{3}H]flunitrazepam-labeled benzodiazepine binding sites or the level of α1 subunit $GABA_A$ receptor mRNA. The number of benzodiazepine binding sites found was in agreement with the number of [^{3}H]flunitrazepam binding sites observed by Zhu et al. (1995). In their study on primary culture of rat CGCs, the maximum number of benzodiazepine binding sites was 59 ± 7 fmol/mg protein and 100 ± 7 fmol/mg protein in the presence of low and high K^+, respectively.

Although down-regulation of $GABA_A$ receptor number is considered a potential mechanism for the development of tolerance in response to prolonged benzodiazepine treatment, a decrease in the number of benzodiazepine binding sites has been observed in only a few studies (for review see Bateson, 2002). As in the present study, most radioligand binding studies have reported no changes in the number of benzodiazepine binding sites following prolonged treatment with positive allosteric modulators of the $GABA_A$ receptor (Bateson, 2002; Uusi-Oukari and Korpi, 2010). In accordance with our results, Follesa et al. (2002) observed no changes in the levels of $GABA_A$ receptor mRNA (α1, α4, γ2L, γ2S) following treatment of CGCs with 10 μM zolpidem for 5 days. In contrast, Holt et al. (1997), who studied the effects of zolpidem treatment in rats, reported that the effects on $GABA_A$ receptor gene expression in the cortex depended on the exposure time: the effect obtained after 7 days (increased level of α4 mRNA) markedly differed from that observed after 14 days (decreased level of α1 mRNA). Decreased expression of α1 $GABA_A$ receptor subunit mRNA was also observed in the prefrontal cortex after treatment of rats with zolpidem for 14 days (Auta et al., 2008).

The results of the present study show that two-day zolpidem treatment did not change the maximum number of [^{3}H]flunitrazepam-labeled benzodiazepine binding sites; these data differ from our previous that demonstrated zolpidem-mediated up-regulation of [^{3}H]flunitrazepam, [^{3}H]muscimol and [^{3}H]TBOB binding sites on recombinant α1β2γ2s $GABA_A$ receptors in HEK 293 cells (Vlainić et al., 2010). There are several possible reasons for this discrepancy. For example, while in the previous study we investigated the effect of drug on one subtype of $GABA_A$ receptors (α1 subunit-containing), the population of $GABA_A$ receptors in CGCs is heterogeneous (Bovolin et al., 1992).

[^{3}H]Flunitrazepam is not a subtype-selective ligand because with benzodiazepine sensitive γ2 subunit-containing receptors, beside α1, α2, α3 and α5 subunit-containing $GABA_A$ receptors are labeled. Thus, it is possible that in our previous study, zolpidem-mediated up-regulation of α1 subtype $GABA_A$ receptors was masked by simultaneous up-regulation of $GABA_A$ receptors containing other alpha subunit isoforms.

Bovolin et al. (1992) reported that the alpha subunit family members α1, α5 and α6 are expressed in granule cells. While the expression of the α5 subunit mRNA was found to be several times lower than that of the α1 subunit, the levels of α4 and especially α2 and α3 mRNAs are very low. Additionally, $GABA_A$ receptors containing α4 and α6 subunits are benzodiazepine- and zolpidem-insensitive (Sieghart, 1995). Moreover, unlike classical benzodiazepines that are non-selective, zolpidem has a very high affinity for receptors containing the α1 subunit, intermediate affinity for receptors that contain α2 or α3, and very low affinity for α5 subunit-containing $GABA_A$ receptors (Arbilla et al., 1985; Depoortere et al., 1986; Sanna et al., 2002; Ci et al., 2007; Korpi et al., 2002). Thus, it does not seem likely that zolpidem-mediated up-regulation of the α1 subtype of $GABA_A$ receptors could compensate by simultaneously causing down-regulation of $GABA_A$ receptors containing other alpha subunit isoforms.

The γ2 subunit together with the α subunit is essential for benzodiazepine binding and pharmacology (Pritchett et al., 1989; Buhr and Sigel, 1997). Cope et al. (2004) demonstrated in vivo that the γ2 subunit is important for the action of zolpidem as well. Therefore, altered expression of this subunit would affect the binding of [^{3}H]flunitrazepam to its binding sites located at the interface of an α and a γ2 subunit (Sieghart, 1995; Buhr and Sigel, 1997). Nevertheless, it has been reported that long-term (7 days) treatment of rats, as well as cultured CGCs (5 days) with zolpidem, had no effect on γ2 mRNA expression (Holt et al., 1997; Follesa et al., 2002).

Thus, as in the present study neither the level of α1 subunit mRNA nor the number of [^{3}H]flunitrazepam-labeled benzodiazepine binding sites changed following two-day zolpidem treatment, we postulate that the expression of the α1 and γ2 subunits was not changed either. This hypothesis is supported in part by the study of Uusi-Oukari et al. (2000). These authors observed down-regulation of α1 mRNA levels in forebrain accompanied with similar down-regulation of α1 polypeptide, which confirmed their hypothesis that there is a tight correlation between α1 mRNA and polypeptide levels.

Thus, we suggest that differences between the results obtained in this study on CGCs and those observed previously on the recombinant α1β2γ2s $GABA_A$ receptors might be explained by differences in the experimental models used. Although the HEK 293 cell line, as a

recombinant expression system, permits an analysis of specific $GABA_A$ receptor subtypes, the regulation of transfected genes may not be the same as in the cultured CGCs that contain a mixture of endogenous genes encoding different $GABA_A$ receptor subunits (Bovolin et al., 1992). Additionally, although HEK 293 cells express many proteins typically found in neurons (Shaw et al., 2002), the expression profile in these cells might still be different due to a lack of a normal neuronal environment, the absence of synapses, the absence of accessory proteins or proteins affecting trafficking of receptors, etc. (Birnir and Korpi, 2007).

It has been suggested that the functional uncoupling of GABA and benzodiazepine binding sites, characterized by a decreased ability of benzodiazepines to potentiate the action of GABA as well as by a decrease in the ability of GABA to potentiate benzodiazepine binding to $GABA_A$ receptors, plays a role in the development of tolerance and dependence to benzodiazepine-like drugs (Gallager et al., 1984). The results obtained in the present study demonstrate that the functional interactions between GABA and benzodiazepine binding sites at $GABA_A$ receptors were diminished in the zolpidem-treated group by approximately 40% (Fig. 3). Although uncoupling of benzodiazepine and GABA binding sites can be caused by drugs that inhibit protein kinase A (PKA), Ali and Olsen (2001) demonstrated that direct phosphorylation of the $GABA_A$ receptor is not involved in the processes of coupling and uncoupling, as mutation of the PKA phosphorylation site did not abolish the effect of chronic benzodiazepine treatment. They suggested that chronic benzodiazepine exposure induces internalization of surface $GABA_A$ receptors into intracellular compartments, where benzodiazepine binding can occur, but its potentiation by GABA is impaired. However, as previously discussed, following chronic diazepam treatment, almost all GABA and benzodiazepine binding sites are expressed on the cell surface, suggesting that chronic exposure to diazepam does not result in internalization of benzodiazepine binding sites (Primus et al., 1996; Perićić et al., 2007). Nevertheless, our results showing that two-day zolpidem treatment produced uncoupling of $GABA_A$ receptors are in accordance with previous reports showing uncoupling of native (Gallager et al., 1984; Hu and Ticku, 1994; Costa and Guidotti, 1996; Brown et al., 1998) and recombinant $GABA_A$ receptors following chronic treatment with classical benzodiazepines (Klein et al., 1994, 1995; Primus et al., 1996; Ali and Olsen, 2001; Švob Štrac et al., 2008) as well as zolpidem (Vlainić et al., 2010).

The observed decreased allosteric linkage between GABA and benzodiazepine binding sites could reflect conformational changes at the binding sites of $GABA_A$ receptor complexes induced by zolpidem at the benzodiazepine binding site. Morlock and Czajkowski (2011) speculated that positioning of the drug at the benzodiazepine binding site and/or positioning of nearby residues induces different downstream allosteric rearrangements. Thus, it remains to be determined whether similar changes are involved in the observed zolpidem-mediated decreased ability of GABA to potentiate binding of benzodiazepine binding site ligands.

Several groups of authors (Hu and Ticku, 1994; Costa and Guidotti, 1996; Brown et al., 1998) have proposed that the changes in allosteric coupling of $GABA_A$ receptor binding sites could be the result of modification from benzodiazepine-sensitive to benzodiazepine-insensitive receptor subtypes and/or changes in receptor function. However, functional uncoupling of GABA and benzodiazepine binding sites following prolonged treatment with benzodiazepines (Klein et al., 1994, 1995; Primus et al., 1996; Perićić et al., 2007; Švob Štrac et al., 2008) or zolpidem (Vlainić et al., 2010) was observed also with recombinant $GABA_A$ receptors with a defined subunit composition. Therefore, we hypothesize that this phenomenon is not associated with the replacement of receptor subunits, as previously suggested. Long-term use of benzodiazepines acting as positive allosteric modulators of GABA action at $GABA_A$ receptors in animals and humans has been associated with molecular modulations of these receptors and probably participate in the mechanisms of tolerance characterized by a decreased ability of drugs to exert their pharmacological effects (for review see Bateson, 2002; Wafford, 2005). However, the molecular mechanisms that underlie tolerance and dependence are still unclear and rather complex (for review see Bateson, 2002; Wafford, 2005). In the present study, we have shown that two-day zolpidem treatment of CGCs expressing $GABA_A$ receptors does not change the maximum number of benzodiazepine binding sites or the expression of $\alpha 1$ mRNA. However, zolpidem treatment could lead to declined GABAergic activity of the receptor. The exact functional consequences of zolpidem-mediated reduction in allosteric linkages between GABA and benzodiazepine binding sites at $GABA_A$ receptors should be determined by additional electrophysiological studies.

Conclusion

These results demonstrate that two-day treatment of CGCs with zolpidem (10 μM) did not induce changes either in the number of benzodiazepine binding sites of $GABA_A$ receptors or in the expression of $GABA_A$ receptor $\alpha 1$ subunit mRNA. As demonstrated by a decreased ability of GABA to stimulate [^{3}H]flunitrazepam binding, two-day exposure of these cells to zolpidem caused functional uncoupling of GABA and benzodiazepine binding sites at $GABA_A$ receptor complexes. If this mechanism is responsible for the development of tolerance following chronic administration of classic benzodiazepines, zolpidem treatment might also induce tolerance.

Conflict of interest

None to declare.

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References

Ali NJ, Olsen RW. Chronic benzodiazepine treatment of cells expressing recombinant GABA(A) receptors uncouples allosteric binding: studies on possible mechanisms. J Neurochem 2001;79:1100–8.

Arbilla S, Depoortere H, George P, Langer SZ. Pharmacological profile of the imidazopyridine zolpidem at benzodiazepine receptors and electrocorticogram in rats. Naunyn Schmiedebergs Arch Pharmacol 1985;330:248–51.

Auta J, Impagnatiello F, Kadriu B, Guidotti A, Costa E. Imidazenil: a low efficacy agonist at alpha1- but high efficacy at alpha5-GABAA receptors fail to show anticonvulsant cross tolerance to diazepam or zolpidem. Neuropharmacology 2008;55:148–53.

Bateson AN. Basic pharmacological mechanisms involved in benzodiazepine tolerance and withdrawal. Curr Pharm Des 2002;8:5-21.

Birnir B, Korpi ER. The impact of sub-cellular location and intracellular neuronal proteins on properties of GABA(A) receptors. Curr Pharm Des 2007;13:3169–77.

Bovolin P, Santi MR, Puia G, Costa E, Grayson D. Expression patterns of gamma-aminobutyric acid type A receptor subunit mRNAs in primary cultures of granule neurons and astrocytes from neonatal rat cerebella. Proc Natl Acad Sci 1992;89:9344–8.

Brown MJ, Wood MD, Coldwell MC, Bristow DR. γ-Aminobutyric $acid_A$ receptor function is desensitized in rat cultured cerebellar granule cells following chronic flunitrazepam treatment. J Neurochem 1998;71:1232–40.

Buhr A, Sigel E. A point mutation in the gamma2 subunit of gamma-aminobutyric acid type A receptors results in altered benzodiazepine binding site specificity. Proc Natl Acad Sci U S A 1997;94:8824–9.

Ci SQ, Ren TR, Ma CX, Su ZG. Modeling of alphak/gamma2 (k = 1, 2, 3 and 5) interface of $GABA_A$ receptor and docking studies with zolpidem: implications for selectivity. J Mol Graph Model 2007;26:537–45.

Cope DW, Wulff P, Oberto A, Aller MI, Capogna M, Ferraguti F, et al. Abolition of zolpidem sensitivity in mice with a point mutation in the $GABA_A$ receptor gamma2 subunit. Neuropharmacology 2004;4:17–34.

Costa E, Guidotti A. Benzodiazepines on trial: a research strategy for their rehabilitation. Trends Pharmacol Sci 1996;71:1232–40.

Crestani F, Martin JR, Mohler H, Rudolph U. Mechanism of action of the hypnotic zolpidem in vivo. Br J Pharmacol 2000;131:1251–4.

DeBlasi A, O'Reilly K, Motulsky HJ. Calculating receptor number from binding experiments using same compound as radioligand and competitor. Trends Pharmacol Sci 1989;10:227–9.
Depoortere H, Zivkovic B, Lloyd KG, Sanger DJ, Perrault G, Langer SZ, et al. Zolpidem, a novel nonbenzodiazepine hypnotic. I. Neuropharmacological and behavioral effects. J Pharmacol Exp Ther 1986;237:649–58.
Follesa P, Mancuso L, Biggio F, Cagetti E, Franco M, Trapani G, et al. Changes in GABA(A) receptor gene expression induced by withdrawal of, but not by long-term exposure to, zaleplon or zolpidem. Neuropharmacology 2002;42:191–8.
Friedman LK, Gibbs TT, Farb DH. gamma-aminobutyric acidA receptor regulation: heterologous uncoupling of modulatory site interactions induced by chronic steroid, barbiturate, benzodiazepine, or GABA treatment in culture. Brain Res 1996;707:100–9.
Gallager DW, Lakoski JM, Gonsalves SF, Rauch SL. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. Nature 1984;308:74–7.
Holt RA, Bateson AN, Martin IL. Chronic zolpidem treatment alters GABA(A) receptor mRNA levels in the rat cortex. Eur J Pharmacol 1997;329:129–32.
Hu XJ, Ticku MK. Chronic benzodiazepine agonist treatment reduces functional uncoupling of the recombinant of the γ-aminobutyric acid-benzodiazepine receptor ionophore complex in cortical neurons. Mol Pharmacol 1994;45:618–25.
Jazvinšćak Jembrek M, Švob Štrac D, Vlainić J, Peričić D. The role of transcriptional and translational mechanisms in flumazenil-induced up-regulation of recombinant GABA(A) receptors. Neurosci Res 2008;61:234–41.
Klein RL, Whiting PJ, Harris RA. Benzodiazepine treatment causes uncoupling of recombinant $GABA_A$ receptors expressed in stably transfected cells. J Neurochem 1994;63:2349–52.
Klein RL, Mascia MP, Harkness PC, Hadingham KL, Whiting PJ, Harris RA. Regulation of allosteric coupling and function of stably expressed γ-aminobutyric acid $(GABA)_A$ receptors by chronic treatment with $GABA_A$ and benzodiazepine agonists. J Pharmacol Exp Ther 1995;274:1484–92.
Korpi ER, Grunder G, Lüddens H. Drug interactions at GABA(A) receptors. Prog Neurobiol 2002;67:113–59.
Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 1992;12:4151–72.
Morlock EV, Czajkowski C. Different residues in the $GABA_A$ receptor benzodiazepine binding pocket mediate benzodiazepine efficacy and binding. Mol Pharmacol 2011;80:14–22.
Oberdoerster J, Rabin RA. Enhanced caspase activity during ethanol-induced apoptosis in rat cerebellar granule cells. Eur J Pharmacol 1999;385:273–82.
Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Pharmacol Rev 2008;60:243–60.
Peričić D, Lazić J, Jazvinšćak Jembrek M, Švob Štrac D, Rajčan I. Chronic exposure of cells expressing recombinant GABAA receptors to benzodiazepine antagonist flumazenil enhances the maximum number of benzodiazepine binding sites. Life Sci 2004;76:303–17.
Peričić D, Jazvinšćak Jembrek M, Švob Štrac D, Lazić J, Špoljarić IR. Enhancement of benzodiazepine binding sites following chronic treatment with flumazenil. Eur J Pharmacol 2005;507:7-13.
Peričić D, Švob Štrac D, Jazvinšćak Jembrek M, Vlainić J. Allosteric uncoupling and upregulation of benzodiazepine and GABA recognition sites following chronic diazepam treatment of HEK 293 cells stably transfected with alpha1beta2gamma2S subunits of GABA(A) receptors. Naunyn Schmiedebergs Arch Pharmacol 2007;375:177–87.
Peričić D, Vlainić J, Švob Štrac D. Sedative and anticonvulsant effects of zolpidem in adult and aged mice. J Neural Transm 2008;115:795–802.
Perrault G, Morel E, Sanger DJ, Zivkovic B. Lack of tolerance and physical dependence upon repeated treatment with the novel hypnotic zolpidem. J Pharmacol Exp Ther 1992;263:298–303.
Primus RJ, Yu J, Xu J, Hartnett C, Meyyappan M, Kostas C, et al. Allosteric uncoupling after chronic benzodiazepine exposure of recombinant γ-aminobutyric $acid_A$ receptors expressed in Sf9 cells: ligand efficacy and subtype selectivity. J Pharmacol Exp Ther 1996;276:882–90.
Pritchett DB, Lüddens H, Seeburg PH. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. Science 1989;245:1389–92.
Roca DJ, Schiller GD, Friedman L, Rozenberg I, Gibbs TT, Farb DH. γ-Aminobutyric acidA receptor regulation in culture: altered allosteric interactions following prolonged exposure to benzodiazepines, barbiturates, and methylxanthines. Mol Pharmacol 1990;37:710–9.
Sanger DJ, Morel E, Perrault G. Comparison of the pharmacological profiles of the hypnotic drugs, zaleplon and zolpidem. Eur J Pharmacol 1996;313:35–42.
Sanna E, Busonero F, Talani G, Carta M, Massa F, Peis M, et al. Comparison of the effects of zaleplon, zolpidem, and triazolam at various GABA(A) receptor subtypes. Eur J Pharmacol 2002;451:103–10.
Shaw G, Morse S, Ararat M, Graham FL. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. FASEB J 2002;16:869–71.
Sieghart W. Structure and pharmacology of γ-aminobutyric acidA receptor subtypes. Pharmacol Rev 1995;47:181–234.
Švob Štrac D, Vlainić J, Jazvinšćak Jembrek M, Peričić D. Differential effects of diazepam treatment and withdrawal on recombinant GABAA receptor expression and functional coupling. Brain Res 2008;1246:29–40.
Uusi-Oukari M, Korpi ER. Regulation of $GABA_A$ receptor subunit expression by pharmacological agents. Pharmacol Rev 2010;62:97-135.
Uusi-Oukari M, Heikkilä J, Sinkkonen ST, Mäkelä R, Hauer B, Homanics GE, et al. Long-range interactions in neuronal gene expression: evidence from gene targeting in the $GABA_A$ receptor β2-α6-α1-γ2 subunit gene cluster. Mol Cell Neurosci 2000;16:34–41.
Vlainić J, Peričić D. Effect of acute and repeated zolpidem treatment on pentylenetetrazole-induced seizure threshold and on locomotor activity: comparison with diazepam. Neuropharmacology 2009;56:1124–30.
Vlainić J, Peričić D. Zolpidem is a potent anticonvulsant in adult and aged mice. Brain Res 2010;1310:181–8.
Vlainić J, Jazvinšćak Jembrek M, Švob Štrac D, Peričić D. The effects of zolpidem treatment and withdrawal on the in vitro expression of recombinant alpha1beta2gamma2s GABA(A) receptors expressed in HEK 293 cells. Naunyn Schmiedebergs Arch Pharmacol 2010;382:201–12.
Wafford KA. $GABA_A$ receptor subtypes: any clues to the mechanism of benzodiazepine dependence? Curr Opin Pharmacol 2005;5:47–52.
Zhu WJ, Vicini S, Harris BT, Grayson DR. NMDA-mediated modulation of gamma-aminobutyric acid type A receptor function in cerebellar granule neurons. J Neurosci 1995;15:7692–701.