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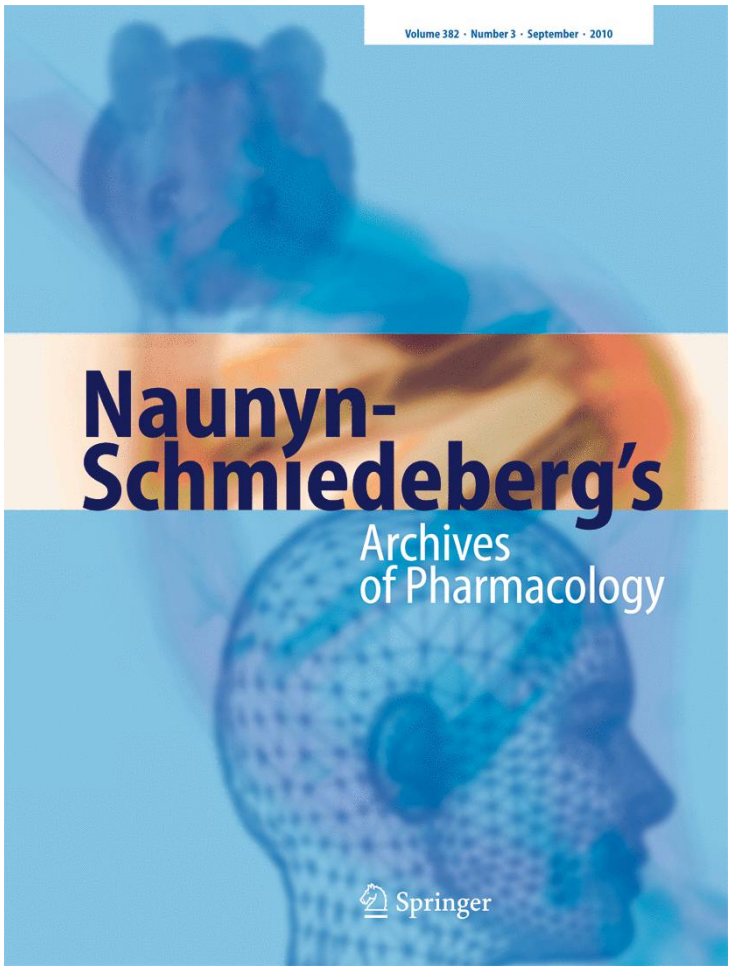

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ORIGINAL ARTICLE

The effects of zolpidem treatment and withdrawal on the in vitro expression of recombinant α1β2γ2s $GABA_A$ receptors expressed in HEK 293 cells

Josipa Vlainić · Maja Jazvinšćak Jembrek · Dubravka Švob Štrac · Danka Peričić

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Abstract Zolpidem, a widely used hypnotic drug which acts through benzodiazepine binding sites, is a positive allosteric modulator of gamma-aminobutyric acid (GABA) action with preferential affinity for $GABA_A$ receptors containing α1 subunit. The pharmacological profile of zolpidem is different from that of classical benzodiazepines. The aim of this study was to find out whether zolpidem treatment triggers adaptive changes in the recombinant α1 subunit-containing $GABA_A$ receptors other than those observed following treatment with classical benzodiazepine—diazepam. Radioligand binding studies showed that 2-day exposure of human embryonic kidney (HEK) 293 cells stably expressing recombinant α1β2γ2s $GABA_A$ receptors to zolpidem (10 μM) up-regulated the maximum number (B_{max}) of [^{3}H]flunitrazepam, [^{3}H]muscimol, and [^{3}H]t-butylbicycloorthobenzoate ([^{3}H]TBOB) binding sites without changing their affinity (K_d), suggesting an increase in total $GABA_A$ receptor number. Semi-quantitative RT-PCR analysis demonstrated increased levels of α1 subunit mRNA, while Western blot demonstrated up-regulated γ2 subunit proteins, suggesting that zolpidem induced de novo synthesis of receptors proteins, at both the transcriptional and translational levels. GABA-induced potentiation of [^{3}H]flunitrazepam binding to membranes obtained from zolpidem-treated cells was markedly reduced, indicating allosteric uncoupling between GABA and benzodiazepine binding sites. The number of benzodiazepine and convulsant binding sites as well as the functional coupling between GABA and benzodiazepine binding sites normalized in 24 h following discontinuation of zolpidem treatment. The results of our in vitro studies suggest that a 2-day exposure of recombinant α1 subunit-containing $GABA_A$ receptors stably transfected in HEK 293 cells to zolpidem induces adaptive changes in this selective $GABA_A$ receptor subtype, which are not substantially different from those obtained after prolonged exposure of cells to high concentrations of diazepam.

Keywords Zolpidem · Recombinant $GABA_A$ receptor · Ligand binding · Gene and protein expression

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Introduction

Gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the central nervous system, mediates its physiological actions via two major classes of GABA receptors, the ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors (Olsen and Sieghart 2008). $GABA_A$ receptors are pentameric heterooligomers composed of various polypeptide subunits (α1-6, β1-3, γ1-3, δ, ε, θ, and π) forming the central ion channel. They possess binding sites for a variety of drugs, such as benzodiazepines, barbiturates, and neurosteroids (Barnard et al. 1998). The functional and pharmacological properties of these receptors are determined by their subunit composition (Korpi et al. 2002).

Zolpidem, a positive allosteric modulator of $GABA_A$ receptors, is a non-benzodiazepine hypnotic which exerts its effects via benzodiazepine allosteric modulatory site on the $GABA_A$ receptor. Unlike classical benzodiazepines which are non-selective, zolpidem has a very high affinity for receptors containing α1 subunit, intermediate affinity for receptors which contain α2 or α3, and lacks interactions at α5 subunit-containing $GABA_A$ receptors (Arbilla et al.

1985; Depoortere et al. 1986; Sanna et al. 2002; Ci et al. 2007). Using knock-in approach, Crestani et al. (2000) also confirmed the preferential affinity of zolpidem for the α1 subtype of $GABA_A$ receptors, while Cope et al. (2004) emphasized in vivo contribution of γ2 subunit to its action. The binding pocket created between α and γ2 subunit is essential for zolpidem binding (Ci et al. 2007). The pharmacological profile of zolpidem is different from that of classical benzodiazepines. Early studies demonstrated its pronounced sedative and mild anxiolytic, anticonvulsant, and myorelaxant properties (Depoortere et al. 1986; Perrault et al. 1990). While studying the mechanisms of zolpidem action, Crestani et al. (2000) suggested that the sedative-hypnotic and anticonvulsant effects of this drug are also due to its action at α1 subunit-containing $GABA_A$ receptors. Studies using sensitive methods showed that zolpidem might be a better anticonvulsant than previously considered (Fahey et al. 2006; Peričić et al. 2008; Vlainić and Peričić 2010).

Long-term administration of non-selective benzodiazepines, which act as full positive allosteric modulators of GABA action at majority of $GABA_A$ receptors, often results in development of tolerance and dependence, phenomena accompanied by different adaptive changes in the GABA system, mainly leading to alterations in receptor expression and/or function. While the observed changes in receptor expression are inconsistent (Wafford 2005), many authors 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) found functional uncoupling of allosteric linkage between GABA and benzodiazepine binding sites, characterized by a decrease in the ability of benzodiazepines to potentiate the action of GABA, as well as by a decrease in the 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 the actions of benzodiazepines. However, in spite of many studies, the molecular mechanisms involved in the development of tolerance and dependence following chronic treatment with these drugs are still poorly known (Bateson 2002; Wafford 2005; Uusi-Oukari and Korpi 2010).

Studies in rodents suggested that unlike benzodiazepines, repeated treatment with zolpidem, a positive allosteric modulator with selectivity for α1 subunit-containing $GABA_A$ receptors, does not produce tolerance and physical dependence (Perrault et al. 1992; Sanger 2004). On the contrary, our study showed development of tolerance and dependence following prolonged zolpidem treatment in mice (Vlainić and Peričić 2009). Therefore, the aim of this study was to find out whether zolpidem treatment and withdrawal trigger adaptive changes in the recombinant α1 subunit-containing $GABA_A$ receptors different from those previously observed following treatment with diazepam.

As a model, we used human embryonic kidney (HEK) 293 cells stably expressing recombinant α1β2γ2s $GABA_A$ receptors, the most common subunit combination of native $GABA_A$ receptors found in the brain (McKernan and Whiting 1996). Related to the origin of HEK 293 cells, Shaw et al. (2002) demonstrated that they contain many proteins typically found in neurons and concluded that these cells resemble more to neurons than to kidney epithelial cells. Radioligand binding studies, semi-quantitative RT-PCR, and Western blot analysis were used to explore the effects of 2-day zolpidem treatment and its withdrawal on the expression and functional coupling of the recombinant α1β2γ2s $GABA_A$ receptors. [^{3}H]muscimol and [^{3}H]flunitrazepam were used to label recognition sites for GABA and benzodiazepines, respectively, while [^{3}H]TBOB was used to label the chloride ion channel, essential for the expression of a functional $GABA_A$ receptor.

Materials and methods

Cell culture

The HEK 293 cell line stably expressing the α1β2γ2s subtype of $GABA_A$ receptor was kindly donated by Dr. David Graham (Sanofi-Synthélabo Research, France). The cells were maintained in 75-cm^2 Falcon flasks according to standard cell culture techniques at 37°C in humidified air with 5% CO_2. They were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Drugs

Zolpidem tartrate [*N*,*N*,6-trimethyl-2-(4-methylphenyl)-imidazo(1,2-a)pyridine-3-acetamide] was a generous gift from Pliva (Zagreb, Croatia). [^{3}H]flunitrazepam (specific activity 87 Ci/mmol) and [^{3}H]t-butylbicycloorthobenzoate ([^{3}H]TBOB, specific activity 20 Ci/mmol) were purchased from Amersham Biosciences UK Ltd. and [^{3}H]muscimol (specific activity 36.5 Ci/mmol) from PerkinElmer (Boston, MA, USA). Unlabeled TBOB was a gift from Professor H. I. Yamamura. All other chemicals used in radioligand binding studies were obtained from commercial sources. Culture medium, antibiotics, and fetal bovine serum were supplied from Invitrogen/Gibco (Grand Island, NY, USA).

Drug treatment

Prior to drug exposure, HEK 293 cells were seeded into new flasks and grown for 3 days in the culture medium. On the day of treatment, medium was removed and replaced

with fresh medium containing zolpidem (final concentration 10 μM) for 2 days in the presence of 1 μM GABA (Sigma, St. Louis, MO, USA). In withdrawal studies, the medium containing zolpidem was replaced after 48-h treatment with the drug-free medium and incubated for additional 24 h in the presence of 1 μM GABA. Zolpidem and GABA were dissolved in distilled water. Control cells were treated with the vehicle in the presence of 1 μM GABA.

Radioligand binding studies

Preparation of the membranes Membranes from stably transfected HEK 293 cells were prepared mainly as previously described (Peričić et al. 2004). The cells were washed with phosphate-buffer saline (PBS), scraped from falcon flasks into ice-cold PBS, and centrifuged at 12,000×*g* for 12 min. The cell pellet was homogenized in 50 mM Tris-citrate buffer, pH 7.4 by ten strokes (up and down) at 1,250 rpm using Teflon pestle and a glass homogenizer, then centrifuged at 200,000×*g* for 20 min. The same procedure (resuspension/centrifugation) was repeated twice. Finally, the pellet was resuspended and stored in aliquots at −20°C. Suspension of the cell membranes was centrifuged on the day of [^{3}H]flunitrazepam and [^{3}H]TBOB binding assay at 200,000×*g* for 20 min. For [^{3}H]muscimol binding assay, membranes were prepared by a modification of the method previously described (Peričić et al. 2007). Briefly, cell membrane suspension was thawed and incubated for 15 min at 4°C in 50 mM Tris-citrate buffer, pH 7.4 containing 0.05% Triton X-100, and then centrifuged at 200,000×*g* for 20 min. The pellet was resuspended in 50 mM Tris-citrate buffer and centrifuged at 17,000×*g* for 10 min three times before use in the [^{3}H]muscimol binding assay.

[^{3}H]Flunitrazepam binding assay Aliquots of the cell membrane preparation (~100 μg protein) were incubated in 50 mM Tris–citrate buffer supplemented with 150 mM NaCl at 4°C for 90 min with addition of varying concentrations of nonradioactive flunitrazepam (ten final concentrations in the range 0.4–50 nM) and a fixed concentration (1 nM) of [^{3}H]flunitrazepam. In stimulation studies, varying concentrations of GABA (1 nM–1 mM) were incubated with [^{3}H]flunitrazepam (1 nM). Nonspecific binding was determined in the presence of 100 μM diazepam. Total assay volume of all binding studies was 0.5 ml. Membrane bound radioactivity was counted on β-scintillation counter (Perkin Elmer, Wallace 1409DSA) after rapid vacuum filtration on Whatman GF/C filters.

[^{3}H]Muscimol binding assay [^{3}H]Muscimol binding to cell membrane preparation (~150 μg protein) was performed at 4°C for 60 min in 50 mM Tris-citrate buffer with addition of 250 mM NaCl. For saturation studies, ten increasing concentrations of nonradioactive muscimol were added (3–150 nM) to a fixed concentration of [^{3}H]muscimol (4 nM). Nonspecific binding was determined in the presence of 1 mM GABA.

[^{3}H]TBOB binding assay Aliquots of the membrane preparation (~100 μg protein) were incubated in the 50 mM Tris-citrate buffer containing 200 mM NaCl at 25°C for 90 min. In saturation studies, ten increasing concentrations of nonradioactive TBOB were added (1–250 nM) to a fixed concentration of [^{3}H]TBOB (8 nM). Nonspecific [^{3}H]TBOB binding was determined in the presence of 100 μM picrotoxin.

Protein concentration determination

In 10 μl of membrane suspension, protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin as standard.

Determination of mRNA levels by semi-quantitative RT-PCR

Total cellular RNA was extracted using High Pure RNA Isolation Kit (Roche) and quantified at 260 nm using a spectrophotometer. Reverse transcription and semi-quantitative PCR were performed as previously described (Jembrek et al. 2008) in a PerkinElmer 9600 Thermocycler. Each RT reaction had two negative controls, a sample without SuperScript II RT and a sample without RNA template, to test for contamination with genomic DNA. As an internal standard in PCR reaction, we used housekeeping gene β-actin whose expression did not change following zolpidem treatment (data not shown). PCR primers for β-actin were TCA CCA ACT GGG ACG ACA TG and TTC GTG GAT GCC ACA GGA CT (Gou et al. 2001) and for $GABA_A$ receptor α1 subunit AGC TAT ACC CCT AAC TTA GCC AGG and AGA AAG CGA TTC TCA GTG GAG AGG (Devaud et al. 1995). Kinetic analysis was performed with each set of primers to assess linearity of PCR products formation with respect to cycle number. The PCR products of both α1 subunit and β-actin gene were measured during the log phase of amplification (β-actin 21, α1 subunit 18 cycles). Samples of the reaction products were separated by electrophoresis on the 1.5% agarose gel and stained with ethidium bromide. The expected lengths of the amplified products were 304 and 602 base pairs for α1 subunit and β-actin genes, respectively. Maximal optical density was obtained with Image Master™ VDS software (Pharmacia). The expression of mRNA of α1 subunit was

normalized to β-actin mRNA expression and compared between control and zolpidem-treated group.

Determination of $GABA_A$ receptor subunit protein levels by Western blot analysis

The preparation of whole-cell protein samples and Western blot analysis were performed mainly as described by Brown and Bristow (1996). The cells were scraped from flasks, centrifuged at 14,000×*g* for 5 min at 22°C, washed, and centrifuged again. The final pellet was resuspended in a reducing and denaturing buffer (5% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 20% glycerol, and 0.1% 0.5 M Tris–HCl/0.4% SDS, pH 6.8) and stored at −20°C. On the day of assay, cell extracts in denaturing buffer were resuspended, boiled for 10 min, and sonicated. Samples were centrifuged at 14,000×*g* for 5 min and then diluted with loading buffer containing 0.1% bromophenol blue. Protein extracts were loaded onto a 5% stacking gel and resolved on a 10% separating gel by SDS–polyacrylamide gel electrophoresis (45 min, 100 V/400 mA, Bio-Rad, USA). After the equilibration of gels in transfer buffer, the proteins were transferred to 0.450-μm pore nitrocellulose membranes (1 h, 100 V/350 mA, 4°C), using a Mini Trans Blot (Bio-Rad). The transfer efficacy was checked by staining the membranes with Ponceau S. Nonspecific sites were blocked by incubation of the membranes in TBS-T buffer (Tris-buffered saline, 0.2% TWEEN 20) containing 5% dried skimmed milk for 1 h at 22°C. Further on, membranes were incubated overnight at 4°C with the primary antibody [polyclonal rabbit antibody (the dilution 1:150 in blocking buffer) directed against rat γ2 subunit of $GABA_A$ receptor (Chemicon)]. The membranes were washed four times during 30 min in TBS-T buffer and exposed to the secondary horseradish peroxidase conjugated anti-rabbit antibody (Santa Cruz Biotechnology) (1:1,000 dilution in blocking buffer) for 1 h at 22°C. Membranes were rewashed four times in TBS-T buffer and then enhanced chemiluminescence (ECL, Perkin-Elmer, USA). Versadoc Model 4000 Imaging system (Bio-Rad) was used to detect the $GABA_A$ receptor γ2 subunit proteins. All experimental data were analyzed within the linear range as determined by loading the increasing amounts of protein samples on the gel. Optical density readings were quantified by Quantity One documentation system and normalized to β-actin protein expression, detected after reprobing the same membranes with primary mouse monoclonal antibody directed against rat β-actin (Sigma). Rat cerebellum extract (Upstate) where the α1β2γ2s combination is the most abundant subtype of $GABA_A$ receptor was used as a positive control. The protein samples extracted from nontransfected HEK 293 cells, which do not significantly express the $GABA_A$ receptor subunits, were used as a negative control.

Data analysis

The analysis of binding data was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). K_d and B_{max} values were obtained by nonlinear regression using the equation for a hyperbola (one binding site): $Y = B_{max} \times X/(K_d + X)$, where K_d is the concentration of ligand required to reach half-maximal binding and B_{max} is the maximum number of binding sites. The percentage of change in [^{3}H]flunitrazepam binding produced by GABA was defined as (specific binding in the presence of GABA/specific binding in the absence of GABA)×100. The enhancement curves, analyzed using the sigmoidal equation, determined the values for half-maximum (EC_{50}) and the maximum enhancement (E_{max}, defined as absolute difference between the top and bottom plateau) of GABA-induced [^{3}H]flunitrazepam binding. Results are presented as means±SEM of at least three independent experiments. The means±SEM for maximum enhancement (E_{max}) of [^{3}H]flunitrazepam binding were determined from individual experiments. Statistical analyses of the results were conducted by one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test or by Student's *t* test, when appropriate. *P* values of less than 0.05 were considered significant.

Results

The effect of zolpidem treatment and its withdrawal on [^{3}H] flunitrazepam binding to membranes from HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors

Zolpidem (10 μM, 48 h) treatment induced an up-regulation of benzodiazepine binding sites at recombinant α1β2γ2s $GABA_A$ receptors stably expressed in HEK 293 cells ($P<0.0001$, ANOVA and Newman–Keuls test). Following the treatment with zolpidem, the cells were incubated for additional 24 h without the drug present. As shown in Fig. 1 withdrawal from zolpidem abolished, the zolpidem induced enhancement of [^{3}H]flunitrazepam binding sites and returned the maximum number of benzodiazepine binding sites to the control values ($P < 0.0001$ versus zolpidem-treated group) (B_{max} values were as follows: control group, 2.505±0.293 pmol/mg protein; zolpidem-treated group, 7.161±0.893 pmol/mg protein; zolpidem-withdrawn group, 2.936±0.392 pmol/mg protein). One-way ANOVA revealed significant differences between these groups [$F(2,9)=25.06$; $P<0.0002$], indicating the significant effect of zolpidem treatment on the maximum number of benzodiazepine binding sites. In addition, the affinity of benzodiazepine binding sites was not affected (K_d values were as follows:

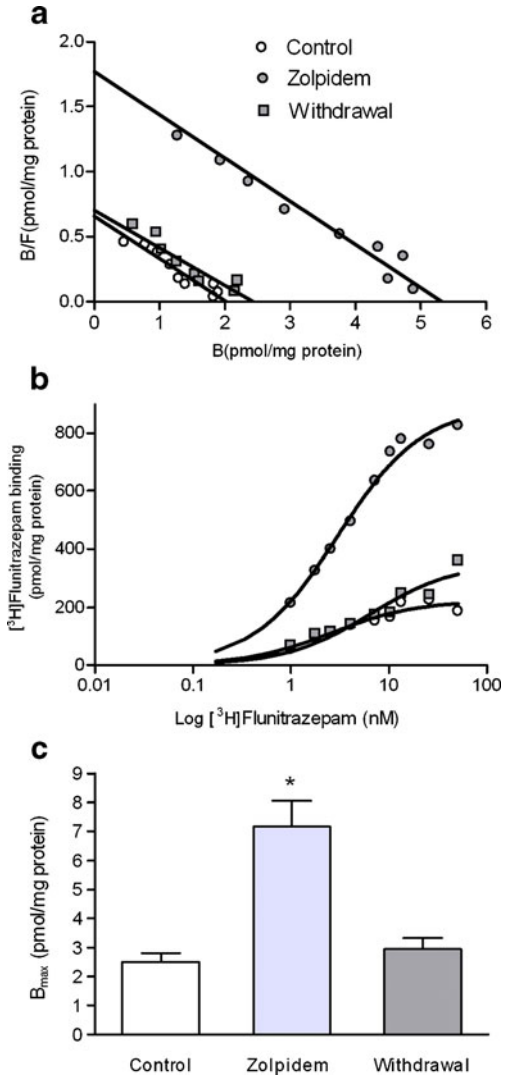

Fig. 1 Scatchard plot (**a**), saturation isotherms (**b**), and the maximum number (**c**) of [^{3}H]flunitrazepam binding sites on the membranes from HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors treated with zolpidem (10 μM, 48 h). Cell membranes were prepared as described in "Materials and methods" and incubated with increasing concentrations of nonradioactive flunitrazepam in the presence of a fixed concentration of [^{3}H]flunitrazepam. Treated samples were always compared with matched controls that were cultured and assayed the same day. The *bars* represent means±SEM (n=3). *P<0.0001 versus control and zolpidem-withdrawn group (ANOVA followed by Newman–Keuls test)

control group, 2.83±0.33 nM; zolpidem-treated group, 3.88± 0.50 nM; and zolpidem-withdrawn group, 2.66±0.61 nM) by the 2-day zolpidem treatment (10 μM).

The effect of zolpidem treatment on [^{3}H]muscimol binding to membrane preparations from HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors

As shown in Fig. 2 and indicated by Student's t test (P< 0.0005), 2-day exposure of HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors to zolpidem (10 μM) enhanced the maximum number (B_{max}) of [^{3}H]muscimol binding sites (control group, 0.742± 0.022 pmol/mg protein; zolpidem-treated group, 2.843± 0.300 pmol/mg protein), without affecting their affinity (K_d; control group, 6.44±0.68 nM; zolpidem-treated group, 7.91±1.10 nM).

The effect of zolpidem treatment and its withdrawal on [^{3}H] TBOB binding to membrane preparations from HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors

The ability of zolpidem treatment (10 μM, 48 h) and its withdrawal (24 h) to affect the parameters of binding sites for convulsants was tested in HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors. The formation of complete, functional $GABA_A$ receptor complex and eventual alterations in the expression ratio of $\alpha1\beta2\gamma2s$:$\alpha1\beta2$ $GABA_A$ receptors were tested in the same way. As shown in Fig. 3, [^{3}H]TBOB bounds to membranes obtained from control and zolpidem-treated cells in a saturable manner. The one-way ANOVA revealed significant differences [F(2,6)=42.53; P<0.0007] in the maximum number (B_{max}) of [^{3}H]TBOB binding sites between the three analyzed groups (control group, 4.237± 0.507 pmol/mg protein; zolpidem-treated group, 11.120± 0.660 pmol/mg protein; and zolpidem-withdrawn group, 4.251±0.710 pmol/mg protein). As indicated by one-way ANOVA and Newman–Keuls test (P<0.001 versus control), exposure of these cells to zolpidem enhanced profoundly the maximum number of [^{3}H]TBOB binding sites, without affecting their affinity (K_d; control group, 47.270±14.490 nM; zolpidem-treated group, 47.740 ± 1.702 nM; and zolpidem-withdrawn group, 41.740± 7.475 nM) (Fig. 3). Following withdrawal from zolpidem treatment, the maximum number of binding sites for convulsants returned to control values within 24 h (P< 0.001 versus zolpidem-treated group).

The effect of zolpidem treatment on the expression of $GABA_A$ receptor $\alpha1$ subunit mRNA

Semi-quantitative RT-PCR analysis for the $\alpha1$ subunit mRNA was performed to assess whether the observed stimulating effect of 2-day zolpidem treatment could be explained by the enhanced transcription of the receptor gene. The amplification products for β-actin and $\alpha1$ subunit from control and zolpidem-treated (10 μM, 48 h) cells had expected molecular size (β-actin 602 bp and $\alpha1$ subunit 304 bp). The negative control (where cDNA was replaced by water) ruled out any contamination in PCR preparations by not showing any amplification. As shown

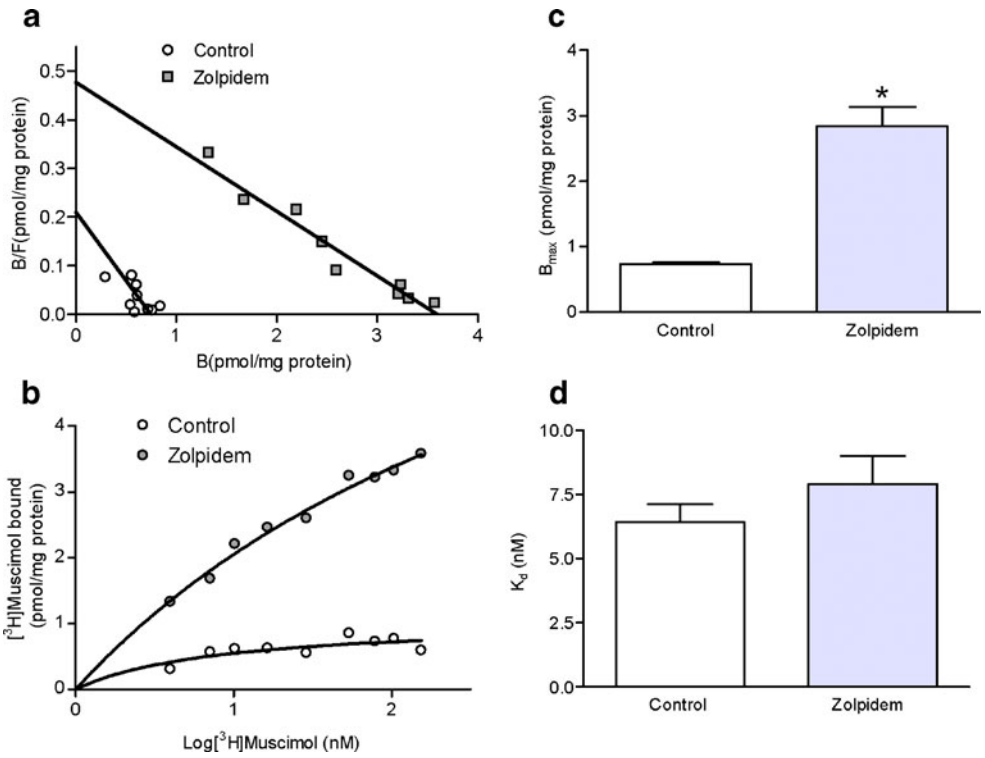

Fig. 2 Scatchard plot (**a**), saturation isotherms (**b**), the maximum number (**c**), and affinity (**d**) of [^{3}H]muscimol binding sites on the membranes from HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors treated with zolpidem (10 μM, 48 h). Cell membranes were prepared as described in "Materials and methods" and incubated with increasing concentrations of nonradioactive muscimol in the presence of a fixed concentration of [^{3}H]muscimol. Treated samples were always compared with matched controls that were cultured and assayed the same day. Results are expressed as means±SEM (n=4). *P<0.0004 versus control group (Student's t test)

in Fig. 4 and indicated by Student's t test (P<0.0001 versus control), zolpidem treatment of HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors up-regulated the $\alpha1$ subunit mRNA levels (54%), suggesting involvement of transcriptional mechanisms in zolpidem-induced changes in the expression of $GABA_A$ receptors.

The effect of zolpidem treatment and withdrawal on γ2-$GABA_A$ receptor subunit protein expression

In order to investigate whether the stimulating effect of zolpidem (10 μM, 48 h) could be explained by de novo protein synthesis and to test whether the enhanced expression of $GABA_A$ receptor subunit mRNA is followed by the corresponding increase in the protein level, the protein level for γ2 subunit of $GABA_A$ receptors was determined. Changes of γ2 subunit protein level following discontinuation (24 h) of zolpidem treatment were also assessed. Figure 5 presents a representative Western blot showing protein band corresponding to γ2 subunit (45 kDa) and β-actin proteins detected in control, zolpidem-treated, vehicle-withdrawn, and zolpidem-withdrawn group as well as positive (rat cerebellum extract) but not at the place of negative control (nontransfected HEK 293 cell extract). The abundance of γ2 subunit protein represented as optical density readings was normalized to the protein expression of corresponding β-actin (42 kDa protein bend). As shown in Fig. 5 and indicated by one-way ANOVA, 2-day exposure of stably transfected HEK 293 cells to zolpidem (10 μM) produced a significant (P<0.001, Newman–Keuls test) up-regulation of γ2 subunit protein levels. At the same time, in the group withdrawn from zolpidem treatment, the protein level for γ2 subunit returned to control level (P < 0.001 versus zolpidem-treated group; Newman–Keuls test). Two control groups showed no differences in the protein levels in this subunit.

The effect of zolpidem treatment and withdrawal on GABA-induced enhancement of [^{3}H]flunitrazepam binding to membranes of HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors

Zolpidem treatment of HEK 293 cells enhanced basal [^{3}H]flunitrazepam binding analogous to that observed in the maximum number of binding sites for benzodiazepines (B_{max}). The addition of GABA (1 nM–1 mM) to membranes obtained from control and zolpidem (10 μM, 48 h) pre-treated cells enhanced [^{3}H]flunitrazepam binding in a concentration-dependent manner. The data were introduced as a percentage of their own basal values and as a maximum enhancement (E_{max}) of [^{3}H]flunitrazepam binding by GABA to better see the differences in the intensity of GABA-induced enhancement of [^{3}H]flunitrazepam binding (Fig. 6). The maximum enhancement (E_{max}) of [^{3}H]flunitrazepam binding produced by GABA in the control group was 80.3±3.0%, indicating that the GABA binding site was functionally coupled to the benzodiazepine binding site. In zolpidem-treated group, E_{max} values were significantly lower (44.2±7.6%). These results indicated that

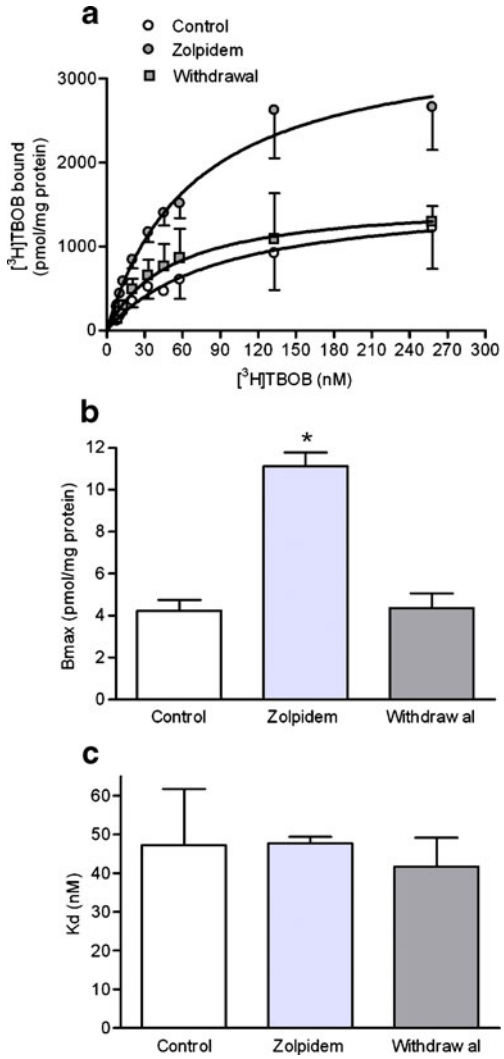

Fig. 3 The effect of zolpidem treatment (10 μM, 48 h) and its withdrawal (24 h) on [^{3}H]TBOB binding to membranes isolated from HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors. Cell membranes prepared as described in "Materials and methods" were incubated with increasing concentrations of nonradioactive TBOB in the presence of a fixed concentration of [^{3}H]TBOB. Results are expressed as means±SEM from three individual experiments performed in duplicate. *P<0.001 (ANOVA and Newman–Keuls test)

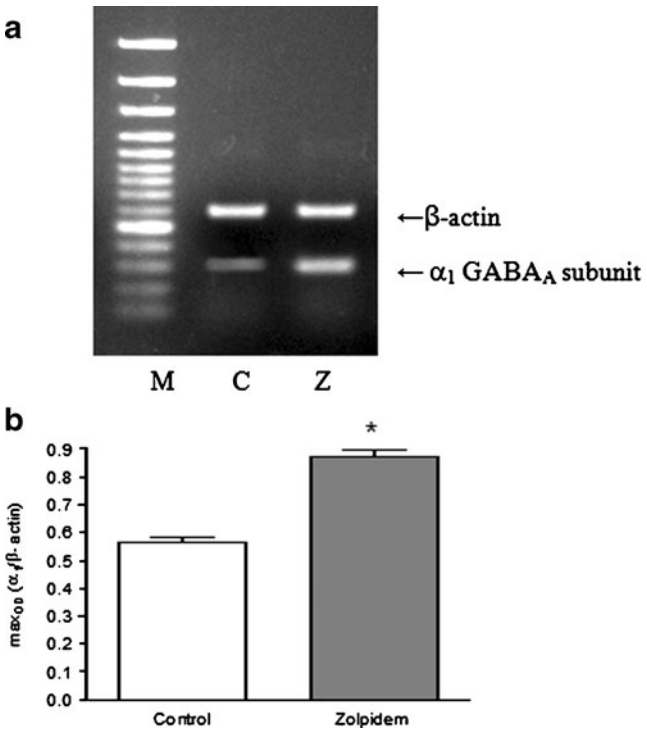

Fig. 4 The effect of zolpidem (10 μM, 48 h) treatment on the expression of $GABA_A$ receptor α1 subunit mRNA: (**a**) representative agarose gel electrophoresis and (**b**) results of RT-PCR analysis. The amount of $GABA_A$ receptor α1 subunit mRNA was determined by semi-quantitative RT-PCR analysis. *M* size marker, *C* products from control cells, *Z* product from zolpidem-treated cells. Amplified products were separated in a 1.5% agarose gel. Densitometric quantification was performed using Image Master. Presented data are expressed as means±SEM from six independent RT-PCR analyses (with PCR performed in triplicate) after three separate preparations of total RNA. *P<0.0001 (Student's t test)

allosteric interactions between GABA and benzodiazepine binding sites were uncoupled by ~55%. A Newman–Keuls test following a significant one-way ANOVA [$F(2,14)$= 13.55; P<0.0005] confirmed the significance (P<0.001) of this effect. As shown by analysis of enhancement curves, there were no differences in concentrations of GABA that produced a half-maximum enhancement of [^{3}H]flunitrazepam binding (EC_{50}) in control and zolpidem-treated group. Following 2-day treatment with zolpidem (10 μM), the cells were incubated for additional 24 h without the drug present. The effect of zolpidem on allosteric uncoupling did not persist in zolpidem-withdrawn group since the maximum enhancement (E_{max}) of [^{3}H]flunitrazepam binding in this group was 76.3±5.1% (Fig. 6).

Discussion

The present study demonstrated that the 2-day exposure of recombinant α1β2γ2s $GABA_A$ receptors stably expressed in HEK 293 cells to hypnotic zolpidem enhanced the maximum number (B_{max}) of [^{3}H]flunitrazepam, [^{3}H]muscimol, and [^{3}H]TBOB binding sites without changing their affinity (K_d), suggesting an increase in the total $GABA_A$ receptor number. This enhancement was accompanied by increased mRNA levels of the α1 subunit, as well as by an increase in the expression of $GABA_A$ receptor γ2 subunit protein. Simultaneously, 2-day zolpidem treatment produced partial uncoupling of allosteric interactions between GABA and benzodiazepine binding sites, as evidenced by a decreased ability of GABA to stimulate [^{3}H]flunitrazepam binding. Zolpidem withdrawal (24 h) normalized the

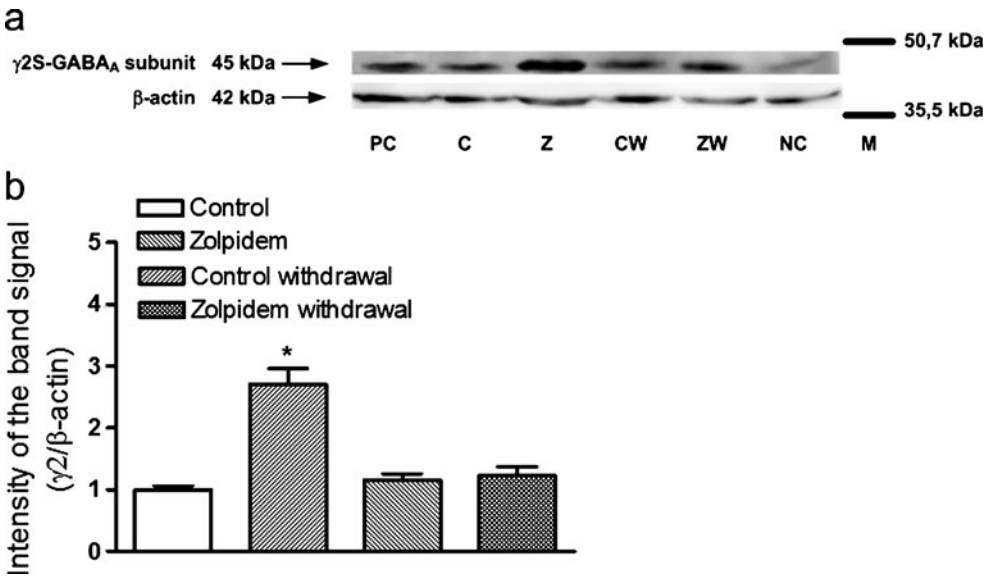

Fig. 5 The effect of zolpidem treatment (10 μM, 48 h) and its withdrawal (24 h) on the protein expression of $GABA_A$ receptor γ2 subunit: **a** representative Western blot showing bands corresponding to γ2 subunit and β-actin proteins and **b** results of semi-quantitative Western blot analysis of γ2 subunit protein expression. *M* size marker, *C* product from control cells, *CW* product from control-withdrawn cells, *Z* product from zolpidem-treated cells, *ZW* product from zolpidem-withdrawn cells, *PC* positive control, *NC* negative control. The protein expression data are presented as means±SEM from four to five independent Western blot analyses after three separate protein preparations. **P*<0.001 versus control group (Student's *t* test)

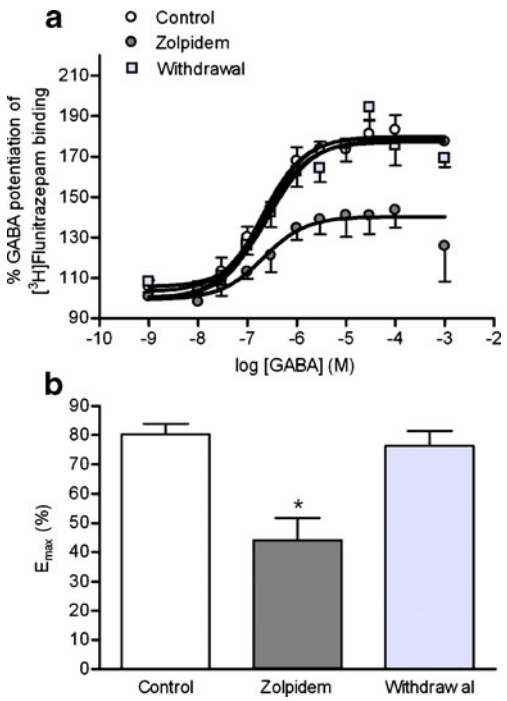

Fig. 6 The effect of zolpidem treatment (10 μM, 48 h) and its withdrawal (24 h) on GABA potentiation of [^{3}H]flunitrazepam binding to membranes of HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors. Data are expressed as percent of their own basal values and the maximum GABA-induced enhancements (E_{max}) of [^{3}H]flunitrazepam binding. Potentiation data were fitted to the sigmoidal equation using GraphPad Prism. The points and bars are means±SEM (n=3–5). **P*<0.001 versus control group (ANOVA and Newman–Keuls test)

number of benzodiazepine and convulsant binding sites, the levels of γ2 subunit proteins, as well as the functional coupling between GABA and benzodiazepine binding sites.

Our previous results obtained on intact HEK 293 cells stably expressing α1β2γ2s $GABA_A$ receptors demonstrated that in the control (Peričić et al. 2007; Jembrek et al. 2008) and in diazepam-treated cells (Peričić et al. 2007), almost all of the [^{3}H]muscimol-labeled GABA binding sites were expressed on the cell surface. Primus et al. (1996), who also studied the localization of $GABA_A$ receptors after chronic diazepam treatment, showed similar results for both GABA and benzodiazepine binding sites on α1β2γ2 subtype of $GABA_A$ receptors expressed in Sf9 cells. The results obtained on primary culture of chick cortical neurons (Calkin and Barnes 1994) showing only a small fraction of intracellular $GABA_A$ receptors in the total pool of cellular receptors were in accordance with the above-mentioned studies. Therefore, we assumed that 2-day zolpidem treatment induced an increase of the cell surface $GABA_A$ receptors. As [^{3}H]TBOB labels the chloride ion channel, which is essential for the expression of a functional $GABA_A$ receptor, our results demonstrating similar zolpidem-induced increases of GABA, benzodiazepine, and convulsant binding sites might be suggesting that the newly expressed $GABA_A$ receptors are functionally active.

The up-regulation of cell surface $GABA_A$ receptors could be the result of a several potential mechanisms, such as an increased synthesis, an enhanced rate of receptor

incorporation into membranes, or a decreased degradation of receptor protein. A general trophic effect of zolpidem treatment on the growth of HEK 293 cells could presumably be excluded because total cellular proteins did not vary between control and zolpidem-treated group of cells (data not shown).

Results of the semi-quantitative PCR analysis showing an increase in the level of α1 subunit mRNA as a result of zolpidem treatment might be suggesting a partial role of transcriptional mechanisms in zolpidem-induced enhancement of $GABA_A$ receptors. It could be expected that the expression of α1 polypeptide was up-regulated as well because there is a tight control between α1 mRNA and α1 polypeptide expression (Uusi-Oukari et al. 2000). Changes in the expression of α1 subunit gene induced by prolonged exposure of receptors to positive modulators of benzodiazepine binding sites at $GABA_A$ receptors are often accompanied with synchronous changes in the expression of γ2 subunit (Follesa et al. 2001; Švob Štrac et al. 2008), which was also the case in our study. It was demonstrated that benzodiazepines (diazepam) regulated α1 (Kang et al. 1994) and γ2 (Holt et al. 1997b) mRNA at the level of transcription, as the changes in transcriptional activity paralleled the changes in their mRNA steady-state levels. The presence of γ2 subunit is required to enable benzodiazepine modulation of the GABA response (Pritchett et al. 1989). Thus, both α1 and γ2 subunits are essential for benzodiazepine binding and pharmacology (Buhr and Sigel 1997). Cope et al. (2004) demonstrated in vivo contribution of γ2 subunit to the zolpidem action reporting the effects of zolpidem being selectively eliminated in mice with a point mutation of the γ2 subunit of the $GABA_A$ receptors. The observed increase in the γ2 subunit proteins along with an increased mRNA for α1 subunit, supports the hypothesis that zolpidem treatment stimulates de novo synthesis of $GABA_A$ receptor proteins by acting at the transcriptional as well as at the translational level. The increase of α1 mRNA and γ2 subunit protein levels was observed in a previous study (Švob Štrac et al. 2008), in which HEK 293 cells expressing recombinant α1β2γ2s $GABA_A$ receptors were exposed to high concentrations of diazepam. On the other hand, Follesa et al. (2002) failed to observe a change of α1 as well as γ2 subunit mRNA in cultured rat cerebellar granule cells treated with 10 μM zolpidem for 5 days. Two studies reported a decreased expression of mRNA encoding the α1 $GABA_A$ receptor subunit in the brain of rats treated with zolpidem for 14 days (Holt et al. 1997a; Auta et al. 2008). Thus, our data related to the expression of benzodiazepine binding sites and $GABA_A$ receptors following treatment with positive modulators of GABA action differ from those obtained with native receptors. Besides the differences in the duration of treatment, there are differences in the experimental models used as well. Unlike native receptors which represent a heterogeneous population of receptors placed in a natural, neuronal environment, recombinant receptors we used are expressed in an artificial milieu and represent a single receptor subtype with a well-defined subunit composition. Additionally, due to pharmacokinetics, the concentration of drug interacting with the receptor is more variable in vivo than it is in vitro. Moreover, it was demonstrated that the regulation of $GABA_A$ receptor subunit expression induced by prolonged benzodiazepine treatment is subunit specific and brain region specific, and occurs at subunit-specific time scales (Uusi-Oukari and Korpi 2010).

It should be mentioned that the 10-μM concentration of zolpidem used in this study, as well as in the study of Follesa et al. (2002), is much greater than normally obtained in humans with therapeutic doses. However, it corresponds well with concentrations which could be achieved in the plasma of drug abusers (3–30-μM concentrations). Namely, as reported by Victorri-Vigneau et al. (2007), zolpidem has a higher abuse potential than previously documented. Ten times lower concentration of zolpidem, as used in our preliminary studies, also enhanced the number of benzodiazepine binding sites, but the enhancement was of borderline significance (results not shown).

As shown in "Results", 2-day exposure of stably transfected HEK 293 cells to zolpidem induced uncoupling of the allosteric linkage between GABA and benzodiazepine binding sites, as evidenced by a decrease in the ability of GABA to potentiate [^{3}H]flunitrazepam binding. Under these conditions, the ability of benzodiazepines to facilitate the action of GABA is decreased as well. A decrease in allosteric coupling at recombinant $GABA_A$ receptors produced by chronic zolpidem treatment (1 μM, 60 h) were also reported by Primus et al. (1996). It was suggested that allosteric uncoupling might be caused by changes in the receptor composition (Hu and Ticku 1994; Costa and Guidotti 1996; Brown et al. 1998). Namely, in the systems with a heterogeneous population of $GABA_A$ receptors, chronic benzodiazepine-induced reduction in the expression of mRNA encoding one $GABA_A$ receptor subunit isoform (mostly α1) is often associated with an enhanced mRNA expression for the other receptor subunit isoform, suggesting $GABA_A$ receptor subunit isoform switching as a possible mechanism involved in adaptive changes induced by prolonged drug treatment (Impagnatiello et al. 1996; Holt et al. 1997a, b; Follesa et al. 2001, 2002). As our stably transfected HEK 293 cells express only one well-defined receptor subtype, replacement of $GABA_A$ receptor subunit isoforms leading to expression of receptors with reduced coupling, as proposed for neurons (Hu and Ticku 1994; Costa and Guidotti 1996; Brown et al. 1998), could

presumably be excluded. As we obtained similar zolpidem-induced enhancements of GABA, benzodiazepine, and convulsant binding sites, the formation of binary $\alpha1\beta2$ forms which do not bind benzodiazepines could most likely be excluded as well, unless accompanied by a parallel formation of $\alpha1\gamma2$s dimers which bind benzodiazepines but are functionally inactive.

Although uncoupling of the benzodiazepine and GABA binding sites could be produced by drugs inhibiting protein kinase A (PKA), mutation of the only PKA phosphorylation site at the recombinant $\alpha1\beta2\gamma2$ receptors indicated that direct phosphorylation of the $GABA_A$ receptor was not involved in the process of coupling and uncoupling (Ali and Olsen 2001). Based on additional studies, the same authors proposed that surface $GABA_A$ receptors became internalized into intracellular compartments where normal benzodiazepine binding could occur, but not potentiation by GABA (Ali and Olsen 2001). However, as previously discussed, Primus et al. (1996) demonstrated that after chronic diazepam treatment, almost all GABA and benzodiazepine binding sites were expressed on the cell surface. Hence, the authors suggested that chronic exposure to diazepam was not a result of the internalization of benzodiazepine binding sites. Our results (Peričić et al. 2007) did not suggest internalization of GABA binding sites either. Thus, the exact mechanism leading to functional uncoupling between GABA and benzodiazepine binding sites remains unknown. Our results showing that 2-day treatment with zolpidem, a selective positive modulator of GABA action at $\alpha1$ subunit-containing $GABA_A$ receptors, produced uncoupling of recombinant $GABA_A$ receptors expressed in HEK 293 cells are in accordance with previous reports showing uncoupling of recombinant $GABA_A$ receptors expressed in nonneuronal cells following chronic treatment with benzodiazepines (Klein et al. 1994, 1995; Primus et al. 1996; Ali and Olsen 2001). While in general allosteric uncoupling between GABA and benzodiazepine binding sites leads to a reduced potency of benzodiazepines, based on our results demonstrating that 2-day zolpidem treatment produced uncoupling along with a pronounced increase of $GABA_A$ receptors, one could not conclude that zolpidem-mediated activity was reduced. Animals and humans treated with non-selective benzodiazepines, acting as full positive allosteric modulators of GABA action at majority of $GABA_A$ receptors, for a prolonged period of time develop tolerance characterized by a decreased ability of benzodiazepines to produce their pharmacological effects. Although it appeared that allosteric uncoupling between the GABA and benzodiazepine binding sites could explain the development of tolerance, the molecular mechanisms underlying benzodiazepine tolerance and dependence are obviously more complex (for reviews, see Bateson 2002).

Withdrawal (24 h) abolished the stimulatory effects of 2-day zolpidem treatment on the expression of benzodiazepine and convulsant binding sites and returned the $\gamma2$ subunit protein to the control levels. It also reversed the functional uncoupling between GABA and benzodiazepine binding sites. On the other hand, in one of our previous studies, the allosteric uncoupling induced by 2-day diazepam treatment of HEK 293 cells expressing $\alpha1\beta2\gamma2$s $GABA_A$ receptors was still present 24 h after the termination of diazepam treatment (Švob Štrac et al. 2008), suggesting that the functional recovery of $GABA_A$ receptors was slower after diazepam compared with zolpidem treatment.

In conclusion, our results showed that 2-day treatment with zolpidem, a positive allosteric modulator with selectivity for $\alpha1$ subunit-containing $GABA_A$ receptors, enhanced the number of $\alpha1\beta2\gamma2$ $GABA_A$ receptors stably expressed in HEK 293 cells and produced functional uncoupling between GABA and benzodiazepine binding sites. Drug withdrawal abolished both effects. Zolpidem induced an increase of mRNA encoding the $\alpha1$ subunit and of the $GABA_A$ receptor $\gamma2$ subunit protein, suggesting that the enhanced number of $GABA_A$ receptors was due to an increased synthesis rather than to a decreased degradation of receptor proteins. Zolpidem triggers adaptive changes in $GABA_A$ receptors in a manner similar to that obtained by high concentrations of diazepam (Peričić et al. 2007; Švob Štrac et al. 2008). Additional electrophysiological studies would be needed to determine the functional consequences of zolpidem-induced enhancement in the number of $GABA_A$ receptors along with the reduced functional coupling between the benzodiazepine and GABA binding sites.

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