

Zolpidem withdrawal induced uncoupling of GABA_A receptors *in vitro* associated with altered GABA_A receptor subunit mRNA expression

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Hypnotic zolpidem produces its effects *via* the benzodiazepine binding site in $\alpha 1$ -containing GABA_A receptors. The aim of the study was to assess the influence of duration of zolpidem treatment and its withdrawal, as well as the role of $\alpha 1$ -containing GABA_A receptors in the development of physical dependence and tolerance. Namely, recombinant receptors can be used to characterize mechanisms involved in different processes in the brain and to delineate the contribution of specific receptor subtypes. To address the influence of chronic zolpidem treatment we exposed HEK293 cells stably expressing $\alpha 1\beta 2\gamma 2S$ recombinant GABA_A receptors for seven consecutive days, while withdrawal periods lasted for 24, 48, 72 and 96 hours. Using radioligand binding studies we determined that chronic zolpidem treatment did not induce changes in either GABA_A receptor number or in the expression of subunit mRNAs. We observed the enhancement of binding sites and upregulated expression of subunit mRNAs only following 96-hour withdrawal. Moreover, zolpidem treatment and its withdrawal (all time points) induced functional uncoupling between GABA and benzodiazepine binding sites in the GABA_A receptor complex. Accordingly, it might be assumed that zolpidem withdrawal-induced uncoupling of GABA_A receptors is associated with altered GABA_A receptor subunit mRNA expression. The results presented here provide an insight into molecular and cellular mechanisms probably underlying adaptive changes of GABA_A receptor function in response to chronic usage and withdrawal of zolpidem and perhaps the observed molecular changes could be linked to the tolerance and dependence produced upon prolonged treatment with other GABAergic drugs.

Key words: zolpidem, chronic treatment, withdrawal, GABA_A receptors, ligand binding, uncoupling, mRNA

INTRODUCTION

Gamma-aminobutyric acid type A receptors (GABA_ARs) are ligand gated ion channels mediating most of the inhibitory actions of the neurotransmitter gamma-aminobutyric acid (GABA) in the central nervous system (Korpi et al. 2002). These receptors are comprised of several subunits ($\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, δ , ϵ , θ , π), mostly being pentamers assembled of two α , two β and one γ subunit (Rudolph et al. 1999). Nevertheless, different subunit composition, especially the presence of a particular α subunit, is essential for the functional

and pharmacological properties of these receptors (Olsen and Sieghart 2008). GABA_ARs are expressed throughout neurons and glial cells and are involved in many brain functions, as well as being the targets for a large number of therapeutics in clinical use: benzodiazepines (BZ), barbiturates, neurosteroids, and general anesthetics.

Zolpidem acts as a positive allosteric modulator at the benzodiazepine binding site of GABA_AR, although chemically it is not a benzodiazepine but an imidazopyridine (Sanger and Zivkovic 1986). Studies on affinity and functional efficacy indicated that zolpidem is selective for GABA_ARs containing $\alpha 1$ subunits, having intermediate affinity for receptors containing $\alpha 2$ or $\alpha 3$ subunits, and lacking interactions with $\alpha 5$ -GABA_ARs (Depoortere et al. 1986, Sanna et al. 2002).

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Genetic studies of knock-in mice confirmed the preferential affinity of zolpidem for the $\alpha 1$ subunit of GABA_ARs (Crestani et al. 2000), consequently exerting a pronounced hypnotic effect. Zolpidem also has mild anxiolytic and myorelaxant effects (Depoortere et al. 1986, Sanger et al. 1996), as well as anticonvulsant activity (Crestani et al. 2000, Fradley et al. 2007, Pericic et al. 2008, Vlainic and Pericic 2010). Acting as hypnotic zolpidem is used in clinics for the treatment of insomnia characterized by inability to initiate sleep. Zolpidem is slowing activity in the brain to allow and maintain sleep (for review see Fitzgerald et al. 2014). Until recently, zolpidem was considered devoid of adverse effects upon short term use and in recommended clinical doses. Nevertheless, there is an increasing body of evidence that long term administration of zolpidem in rodents induces development of tolerance and dependence (Auta et al. 2008, Vlainic and Pericic 2009, Murphy et al. 2011, Wright et al. 2014); and numerous cases of zolpidem abuse and dependence have been reported in humans, especially those having a history of drug and/or alcohol abuse (for review see Victorii-Vigneau et al. 2013). Recent paper summarized current knowledge on zolpidem's action underlying the distinct role of different GABA_ARs in dependence (Fitzgerald et al. 2014). Namely, the development of tolerance and dependence following repeated zolpidem treatment, and the presence of withdrawal syndrome are likely accompanied with different neuroadaptive changes in the GABAergic system, mainly leading to alterations in receptor expression and/or function (for review see Uusi-Oukari and Korpi 2010). Despite many studies, the molecular mechanisms involved in the development of tolerance to the actions of zolpidem are still unknown. Since it is indicated that changes in GABAergic system are dose and time dependent (for review see Uusi-Oukari and Korpi 2010) it is of importance to study different treatment time points. In the current study we investigated the effects of chronic zolpidem treatment (7 days) and different withdrawal periods (24, 48, 72 and 96 h) on the number and on the expression of GABA_AR subunit mRNAs. The study is conducted on recombinant $\alpha 1\beta 2\gamma 2S$ GABA_ARs (the most abundant receptor subtype) stably expressed on the surface of HEK 293 cells. Shaw and colleagues (2002) showed that HEK 293 cells express many proteins typically found in neurons, although the transcription of transfected genes encoding GABA_AR subunits could be

differently regulated than *in vivo* or in primary neuronal culture where endogenous genes are involved as well. Thus, well-defined subunit composition of the recombinant receptors offers some advantages, e.g. homogenous population of receptors, well defined receptor subtype, which are at the same time limitation factors.

METHODS

Cell culture

The human embryonic kidney (HEK) 293 cell line stably expressing the $\alpha 1\beta 2\gamma 2S$ subtype of GABA_A receptor was kindly donated by Dr. David Graham (Besnard et al. 1997). The cells were maintained according to standard cell culture techniques in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37°C in humidified air with 5% CO₂. Culture medium, antibiotics, fetal bovine serum and other chemicals were supplied from commercial suppliers.

Drug treatment

Cells were seeded into new flasks and grown for three days prior to exposure to drugs (zolpidem (N,N,6-trimethyl-2-(4-methylphenyl)-imidazo(1,2-a)pyridine-3-acetamide) was a generous gift from Pliva, Zagreb, Croatia). Control cells were treated with 1 μ M GABA and vehicle (zolpidem and GABA were dissolved in distilled water) and underwent withdrawal as well. Since we studied the effect of the drug during seven consecutive days the medium containing zolpidem (10 μ M) and GABA (1 μ M) was replaced every third day with fresh medium. In withdrawal studies the medium containing zolpidem was replaced with drug-free medium at the day withdrawal started and incubated for additional period of time (24, 48, 72 or 96 h, respectively) in the presence of 1 micromolar GABA.

In order to address the issue of prolonged culturing to the changes in the maximum number of benzodiazepine binding sites, the allosteric linkage between GABA and benzodiazepine binding sites at the recombinant GABA_ARs, and the expression of subunit mRNAs, there were a groups of cells cultured

Table I

Primer sequences and number of cycles used for PCR amplification			
Gene	Sequence description (5' – 3')	Cycle numbers	Amplicon size (bp)
β-actin	TCA CCA ACT GGG ACG ACA TG	24, 25	150
	TTC GTG GAT GCC ACA GGA CT		
α1	AGC TAT ACC CCT AAC TTA GCC AGG	20, 21	304
	AGA AAG CGA TTC TCA GTG GAG AGG		
β2	TGA GAT GGC CAC ATC AGA AGC AGT	17, 18	317
	TCA TGG GAG GCT GGA GTT TAG TTC		
γ2S	AAG AAA AAC CCT GCC CCT ACC ATT	20, 21	336
	TTC GTG AGA TTC AGC GAA TAA GAC		

with the vehicle for seven days that experienced 24, 48, 72 and 96 hours of vehicle withdrawal, respectively. Since we found no changes in the parameters assessed in this study in the control withdrawal groups the results obtained are not presented. Moreover, 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 as well as control- and zolpidem- withdrawn group of cells (data not shown).

Radioligand binding studies

Preparation of the membranes. Membranes from stably transfected HEK293 cells were prepared mainly as described elsewhere (Pericic at al. 2005). The cells were washed with phosphate-buffer saline, scraped from flasks and centrifuged at 12 000×g for 12 min. The cell pellet was homogenized in 50 mM Tris-citrate buffer (pH 7.4) by 10 strokes at 1000×g using teflon pestle and a glass homogenizer, and then centrifuged at 200 000×g for 20 min. The same re-suspension/centrifugation procedure was repeated twice. 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 binding assay at 200 000×g for 20 min.

[³H]flunitrazepam binding assay. Aliquots of the cell membrane preparation (~100 µg protein) were

incubated in a 50 mM Tris-citrate buffer supplemented with 150 mM NaCl at 4°C for 90 min (Pericic at al. 2005) with the addition of varying concentrations of non-radioactive flunitrazepam (ten final concentrations in the range 0.4–50 nM) and a fixed concentration (1 nM) of [³H]flunitrazepam ([³H]flunitrazepam (specific activity 87 Ci/mmol) was purchased from Amersham Biosciences UK Ltd.). In stimulation studies, varying concentrations of GABA (1 nM–1 mM) were incubated with [³H]flunitrazepam (1 nM). Non-specific binding was determined in the presence of 100 µM diazepam. Total assay volume of all binding studies was 0.5 ml. The radioactivity bound to membranes was counted on a LSC counter (Wallace 1409DSA) after a rapid vacuum filtration on Whatman GF/C filters.

Protein determination. Using bovine serum albumin as a standard, protein concentration was determined in 10 µl samples of membrane suspension.

Conventional RT-PCR

Total cellular RNA was extracted using a ReliePrep RNA Cell Miniprep System (Promega, Germany) and quantified at 260 nm using spectrophotometer (NanoDrop, Thermo Scientific). Reverse transcription and semi-quantitative PCR were performed as previously described (Jazvinscak Jembrek at al. 2012). Briefly, together with random hexadeoxynucleotide primers (2.5 µM), total RNA (1 µg) was denatured at 65°C for 5 min,

Table II

The dissociation constant (K_d) of benzodiazepine binding sites following zolpidem treatment and its withdrawal at recombinant GABA _A R	
	Affinity of [³ H]flunitrazepam binding sites (nM)
Control	3.72±0.44
Zolpidem 7 days	4.49±0.61
+ withdrawal 24 h	4.51±0.56
+ withdrawal 48 h	4.05±0.60
+ withdrawal 72 h	2.98±0.95
+ withdrawal 96 h	2.57±0.30

and the first strand of cDNA was synthesized by adding the following reagents: reverse transcription buffer (Invitrogen), 0.5 mM dNTPs (Takara, Japan), 40 U of RNase-inhibitor (Roche, USA) and 200 U of SuperScript II reverse transcriptase (Invitrogen, USA). 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 RNA template to test for contamination with genomic DNA. The resulting cDNA (1:10 dilution) was amplified using EmeraldAmp MAX PCR Master Mix (Takara, Japan) with added primers (0.2 μM). The reactions were performed for a determined number of cycles (cDNA was amplified and analyzed in two consecutive cycles in the logarithmic phase of PCR reaction; Table I) each consisted of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 7 min. The reaction products were separated by electrophoresis on a 1.5% agarose gels and visualized by EtBr staining. Maximal optical density was obtained using the ImageJ software (NIH, USA). The expression of GABA_AR subunits mRNA was normalized to the reference gene β-actin mRNA expression and compared between control and treated groups.

Data analysis

The analysis of binding data was performed using the GraphPad Prism (GraphPad Software, USA). The values K_d and B_{max} 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 concentra-

tion of ligand required to reach half-maximal binding and B_{max} is the maximum number of binding sites. The percentage of change in [³H]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 [³H]flunitrazepam binding.

Statistical evaluation was performed with one-way analysis of variance (ANOVA) followed by a *post-hoc* Dunnett's multiple comparison test. All data are expressed as means±SEM of at least three independent experiments performed in duplicate. *P*-values of equal or less than 0.05 were considered significant.

RESULTS

The number of benzodiazepine binding sites following chronic zolpidem treatment and its withdrawal in HEK 293 cells stably transfected with α1β2γ2S subunits of GABA_ARs

Chronic zolpidem treatment (10 μM, 7 days) and its withdrawal (24, 48, 72 or 96 h, respectively) were studied with respect to the number and the affinity of benzodiazepine binding sites in the recombinant α1β2γ2S GABA_ARs. Control cells were treated with the vehicle and GABA (1 μM). In order to assess the possible influence of prolonged culturing and withdrawal on the maximum number of [³H]flunitrazepam binding sites in the GABA_AR complex, the control groups of cells

also experienced withdrawal. The experiments revealed no changes in the maximum number of benzodiazepine binding sites and the dissociation constant between control groups (data not shown).

Saturation isotherms and Scatchard plots of binding studies are shown in Figure 1. The maximum number of benzodiazepine binding sites (B_{max}) in the group treated with zolpidem for 7 days (5.32 ± 0.82 pmol/mg protein) was unchanged when compared to the control

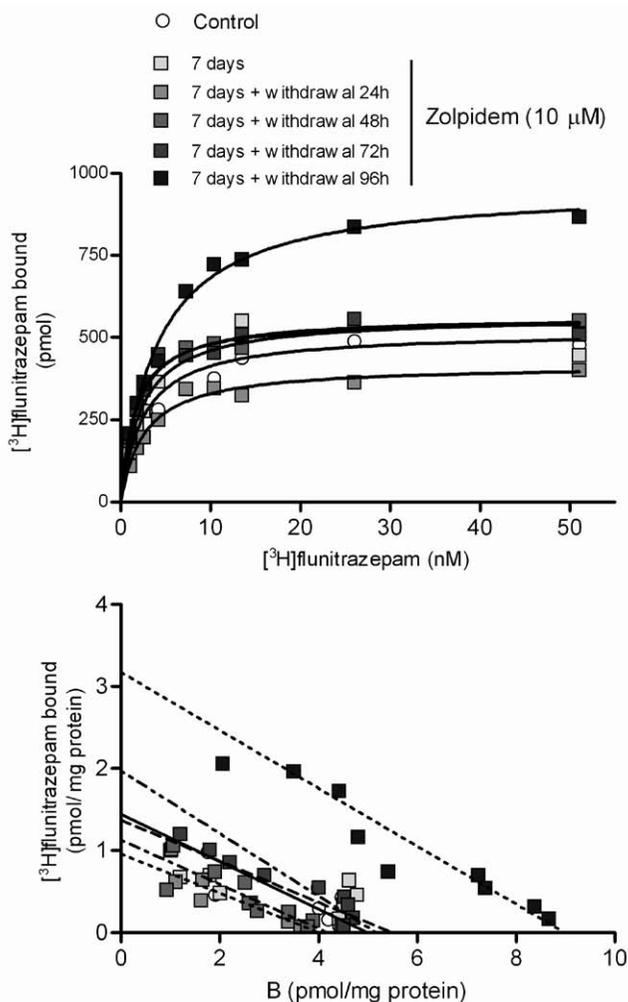


Fig. 1. The effect of zolpidem treatment and different withdrawal periods on saturation isotherms and Scatchard plots of $[^3\text{H}]$ flunitrazepam binding sites on the membranes of HEK 293 cells stably transfected with $\alpha 1\beta 2\gamma 2\text{S}$ subunits of GABA_A Rs. Cell membranes of zolpidem ($10 \mu\text{M}$, seven days) treated and withdrawn (24, 48, 72 and 96 hours) groups were incubated with increasing concentrations of non-radioactive flunitrazepam (0.3–50 nM) in the presence of 1 nM $[^3\text{H}]$ flunitrazepam. Control cells were treated with the vehicle and 1 micromolar GABA as treated groups.

group (4.91 ± 0.82 pmol/mg protein). No change in the maximum number of benzodiazepine binding sites was also observed for withdrawal periods that lasted 24- (4.08 ± 0.30 pmol/mg protein), 48- (4.79 ± 0.64 pmol/mg protein) and 72-hours (4.90 ± 0.33 pmol/mg protein). On the other hand, the maximum number of benzodiazepine binding sites was augmented in the group where the withdrawal period lasted 96 hours (8.67 ± 0.99 pmol/mg protein). One-way ANOVA revealed the significance between the above mentioned groups ($F_{5,16}=4.83$; $P < 0.01$) (Fig. 2). Namely, in the group where zolpidem treatment was withdrawn for 96 hours the maximum number of $[^3\text{H}]$ flunitrazepam binding sites was significantly augmented ($P < 0.05$, *post hoc* Dunnett's Multiple Comparison test) when compared to the control group, but also when compared to zolpidem treated and other zolpidem-withdrawn groups (24, 48 and 72 h, respectively). Upregulation of maximum number of benzodiazepine binding sites following 96 h zolpidem withdrawal was observed when lower zolpidem concentration ($1 \mu\text{M}$) was applied but the results were at the border of significance and thus not shown.

K_d is the equilibrium dissociation constant or binding affinity constant and is the reciprocal value of the affinity. The affinity of benzodiazepine binding sites in zolpidem treated and withdrawn groups remained unchanged (*versus* control group) although one could notice that the dissociation constant of $[^3\text{H}]$ flunitrazepam binding sites is slightly decreased across groups withdrawn from zolpidem especially when withdrawal lasted 96 hours (Table II).

The expression of mRNA of GABA_A R subunits following zolpidem treatment and different withdrawal periods

In order to assess the influence of chronic zolpidem treatment ($10 \mu\text{M}$, 7 days) and its withdrawal (24, 48, 72 or 96 h, respectively) on the expression of GABA_A R subunit mRNA, the amount of $\alpha 1$, $\beta 2$ and $\gamma 2\text{S}$ mRNA was determined by conventional RT-PCR analysis (see sequence description in Table I). The amplification products for the housekeeping gene β actin and receptor subunits had the expected molecular size. Since there was no product in the negative control, the contamination of RNA samples and/or PCR preparation was ruled out. In addition, to assess the influence of prolonged culturing the control groups of cells also underwent withdrawal (control groups were cultured with vehicle

for seven days and then 24, 48, 72 and 96 hour withdrawal). The data for these control groups are not shown since there were no changes in the expression level for the $\alpha 1$, $\beta 2$ and $\gamma 2S$ mRNAs between them.

As shown in Figure 3, the mRNA levels for all three subunits ($\alpha 1$, $\beta 2$ and $\gamma 2S$) were not changed either by zolpidem treatment (7 days, 10 μM) or by withdrawal periods that lasted for 24, 48, or 72h, respectively. Unchanged levels of $\beta 2$ subunit mRNA were observed also for 96-hours withdrawal period in comparison to the control group. On the other hand, statistical analysis using one-way ANOVA revealed significant differences for $\alpha 1$ ($F_{5,71}=3.775$; $P=0.0044$) and $\gamma 2S$ ($F_{5,63}=2.991$; $P=0.017$) but not for $\beta 2$ ($F_{5,60}=0.813$; $P=0.544$) mRNA levels within the zolpidem treated and withdrawal groups. Namely, mRNAs for $\alpha 1$ ($P<0.01$, *post hoc* Dunnett's Multiple Comparison test) and $\gamma 2S$ ($P<0.05$, *post hoc* Dunnett's Multiple Comparison test) subunits were significantly increased following a 96-hour withdrawal period when compared to the mRNA in the control group.

GABA induced enhancement of [3H] flunitrazepam binding to recombinant GABA $_A$ Rs following zolpidem treatment and different withdrawal periods

The effect of chronic zolpidem treatment (7 days, 10 μM) and its withdrawal (24, 48, 72 and 96 h) on GABA potentiation of [3H]flunitrazepam binding as a measure of degree of allosteric coupling between the GABA and benzodiazepine binding site at GABA $_A$ R complex was studied at membranes of HEK 293 cells stably transfected with $\alpha 1\beta 2\gamma 2S$ recombinant GABA $_A$ Rs. Control cells were treated with the vehicle and GABA (1 μM). Moreover, the control groups of cells also underwent withdrawal and since there were no changes in any parameters assessed the data obtained are not shown.

One-way ANOVA followed by Dunnett's multiple comparison test revealed significant differences between these groups ($F_{5,16}=30.90$; $P=0.0001$). The addition of GABA (1 nM–1 mM) to membranes obtained from control and zolpidem treated and withdrawn cells enhanced [3H]flunitrazepam (1 nM) binding in a concentration dependent manner. On the other hand, analyses of the enhancement curves (Fig. 4A) for the control, zolpidem treated and withdrawal groups did not show differences in the concentrations of GABA that produced a half-

maximum enhancement of [3H]flunitrazepam binding (EC_{50}). In order to point out the differences in the intensity of GABA-induced enhancement of [3H]flunitrazepam binding the data are presented as the percentage of their own basal values (Fig. 4A) and as the maximum enhancement (E_{max}) (Fig. 4B).

The maximum enhancement of [3H]flunitrazepam binding produced by GABA in the control group was $64.3\pm 1.8\%$ indicating that the GABA binding sites were functionally coupled to the benzodiazepine binding sites. In the zolpidem treated group the maximum enhancement of benzodiazepine binding ($53.7\pm 2.4\%$) was significantly ($P<0.05$) lower compared to the control group. As shown in Figure 4, the results obtained indicated that zolpidem withdrawal (24, 48, 72 and 96 h) also leads to a significant decline ($P<0.01$ and $P<0.05$) in the level of allosteric coupling between GABA and benzodiazepine binding sites at GABA $_A$ R complex having maximum enhancements of app 53, 52, 47 and 33%, respectively.

Changes in the level of allosteric coupling were observed when the cells were treated with 1 micromo-

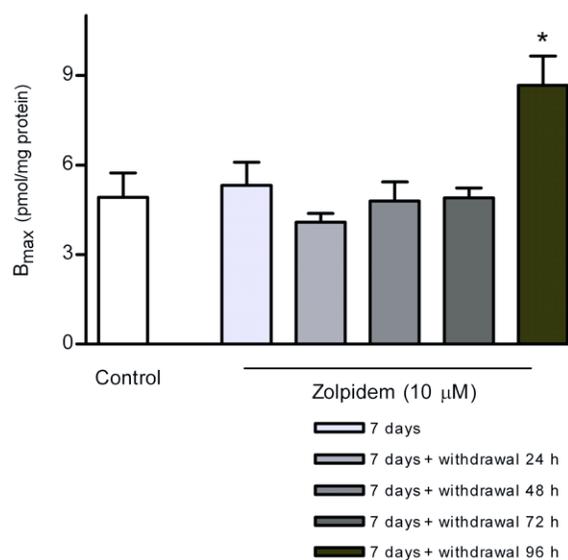


Fig. 2. The effect of zolpidem treatment and different withdrawal periods on the maximum number (B_{max}) of [3H]flunitrazepam binding sites on the membranes of HEK 293 cells stably transfected with $\alpha 1\beta 2\gamma 2S$ subunits of GABA $_A$ Rs. Cell membranes of zolpidem (10 μM , seven days) treated and withdrawn (24, 48, 72 and 96 hours) groups were incubated with increasing concentrations of non-radioactive flunitrazepam (0.3–50 nM) in the presence of 1 nM [3H]flunitrazepam. B_{max} was obtained by nonlinear regression. The results are expressed as means \pm SEM ($n=3$). * $P<0.05$ versus control group (ANOVA followed by the Dunnett's test).

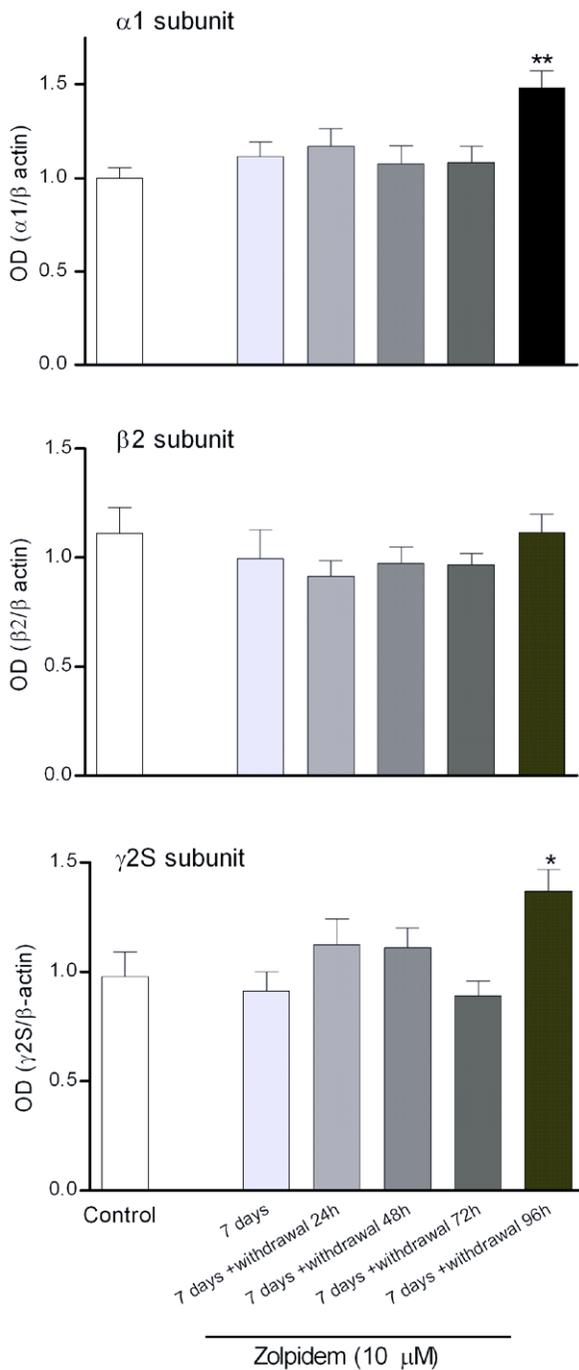


Fig. 3. The effect of zolpidem treatment and different withdrawal periods on the expression of GABA_AR subunit mRNAs: $\alpha 1$, $\beta 2$ and $\gamma 2S$. The amount of GABA_AR subunits mRNA was determined by conventional RT-PCR analysis. The maximal optical density of the subunits mRNA expression was normalized to the expression of reference gene β -actin. Data are expressed as means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ versus control group (ANOVA and Dunnett's test).

lar zolpidem concentration as well, although they were at the border of significance (data not shown).

DISCUSSION

To address the timeframe of the changes induced by chronic zolpidem treatment and withdrawal we exposed cells stably expressing recombinant GABA_ARs to zolpidem (10 μ M) for seven days. Withdrawal periods lasted 24, 48, 72 and 96 hours, respectively. Our results demonstrate that zolpidem treatment and 24-, 48- and 72-hour withdrawal produced no changes in the number of [³H]flunitrazepam binding sites while following 96-hour withdrawal there was an increase in the number of benzodiazepine binding sites in our system (Fig. 1). Similar was observed when the cell culture was treated with 1 micromolar zolpidem upon the same treatment regime but the results were on border of significance (data not shown).

When we related obtained with the previous results, we assumed that detected [³H]flunitrazepam binding sites are probably expressed on the cell surface (Pericic at al. 2007, Svob Strac at al. 2008, Vlainic at al. 2010). There is also no change in the levels of $\alpha 1$, $\beta 2$ and $\gamma 2S$ subunit mRNAs following zolpidem treatment and withdrawal that lasted 24, 48 or 72 hours. Follesa and colleagues (2002) showed that long-term exposure of rat cerebellar granule neurons to zolpidem (10 μ M) does not produce any significant changes in the abundance of mRNA for $\alpha 1/4$, $\beta 1/2/3$ and $\gamma 2$ subunits. Moreover, in several animal studies seven-day zolpidem treatment in rats had no effect on $\alpha 1$ and $\gamma 2$ mRNA expression (Holt at al. 1997, Wright at al. 2014).

Altered number of binding sites for a particular drug, e.g. benzodiazepines, could account for changes in drug action, for instance the need for a greater amount of the drug in order to produce the needed action in the same proportion. Namely, 10 days of zolpidem treatment produced tolerance to its anticonvulsant, hypothermic and ataxic effects as well as withdrawal-like symptoms after drug termination (Auta at al. 2008, Vlainic and Pericic 2009, Wright at al. 2014). Although the most persuasive premise for decreased sensitivity of GABA_ARs after prolonged benzodiazepine treatment is a downregulation of receptor number, this is not supported within the literature (for review see Uusi Oukari and Korpi 2010 and Vinkers and Olivier 2012). Most studies demonstrated no modifications following pro-

longed exposure of cells or animals (Gallagher et al. 1984, Lewin et al. 1989, Galpern et al. 1991, Primus et al. 1996, Toki et al. 1996) and we observed a similar result within our experiments. In contrast, Toki and colleagues (1996) observed an increase in the maximum number of benzodiazepine binding sites at 42 hr after diazepam withdrawal. In addition, 4 days after discontinuation of clonazepam (7 days) administration benzodiazepine binding *in vivo* and *in vitro*, as well as TBPS binding, were increased (Galpern et al. 1991). Changes in receptor number following zolpidem 96-hour withdrawal could be a result of interference at several levels: increased subunit mRNA transcription, decreased subunit degradation, and/or increased expression of helper

GABA_AR-associated proteins (GABARAP, BIG2, PRIP, gephyrin and/or radixin) (for review see Vinkers and Olivier 2012). In the group where withdrawal from prolonged zolpidem treatment lasted 96 hours we observed upregulation of $\alpha 1$ and $\gamma 2S$ subunit mRNA levels while $\beta 2$ mRNA remained at control levels. Although mRNA is an intermediate in protein synthesis, we assume that the expression of $\alpha 1$ and $\gamma 2S$ polypeptides is also upregulated since it has been shown that mRNA expression correlates with polypeptide expression (Uusi-Oukari et al. 2000). Synchronous changes in the expression of $\alpha 1$ and $\gamma 2$ subunits after prolonged benzodiazepine treatment have been observed in previous studies (Follesa et al. 2002, Biggio et al. 2003, Svob Strac et al. 2008). For

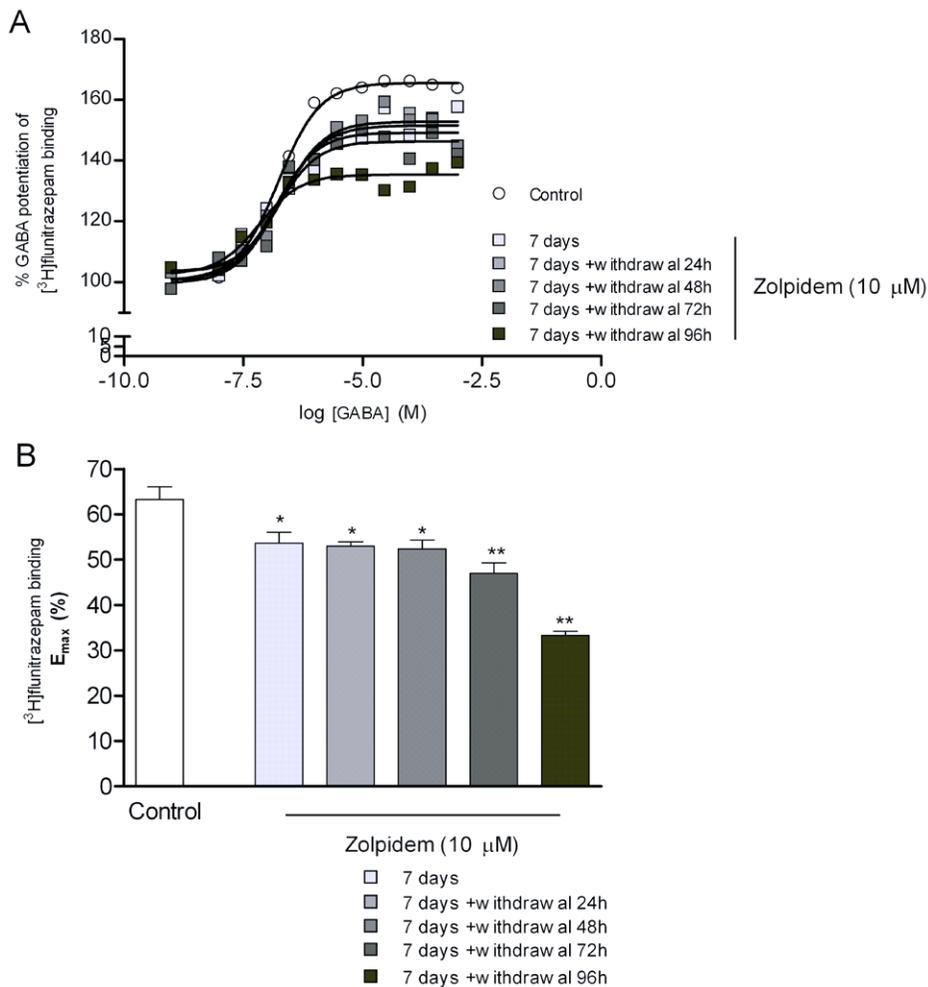


Fig. 4. The effect of chronic zolpidem treatment and different withdrawal periods on GABA potentiation of [³H]flunitrazepam binding to membranes of HEK 293 cells stably transfected with $\alpha 1\beta 2\gamma 2s$ subunits of GABA_ARs. Data are expressed as percent of their own basal values (A) and as the maximum GABA-induced enhancements (B; E_{max}) of [³H]flunitrazepam binding. The points (error bars not shown due to clarity) and bars are means \pm SEM ($n=3-5$). * $P<0.05$ ** $P<0.01$ versus control group (ANOVA and Dunnett's test).

$\alpha 1$ (Kang et al. 1994) and $\gamma 2$ (Holt et al. 1997) subunits the comparative transcriptional activity and changes in mRNA steady-state levels following prolonged benzodiazepine exposure have been detected. Since both $\alpha 1$ (but also $\alpha 2,3,5$) and $\gamma 2$ subunits are essential for benzodiazepine binding and pharmacology (Rudolph et al. 1999) one can speculate that the increase in the maximum number of benzodiazepine binding sites occurred probably due to the partial formation of $\alpha\gamma$ binary forms in the group withdrawn from zolpidem treatment for 96 hours.

The observed increase in the mRNA for $\alpha 1$ and $\gamma 2$ subunits supports our assumption that 96-h withdrawal from prolonged zolpidem treatment stimulates *de novo* synthesis of GABA_AR proteins. Supporting this, it was suggested that the process of GABA_AR synthesis has at least some importance in possible alterations in subunit expression since the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D abolished the effects of chronic diazepam (Pericic et al. 2007) and flumazenil (Jazvinscak Jembrek et al. 2008) treatment.

In our experimental system chronic zolpidem treatment (10 μ M) produced partial uncoupling of allosteric interactions between benzodiazepine and GABA binding sites as evidenced by a decreased ability of GABA to stimulate [³H]flunitrazepam binding. It should be mentioned that similar results were also obtained with lower (1 μ M) zolpidem concentration (data not shown). The maximum enhancement of [³H] flunitrazepam binding produced by GABA was diminished in groups withdrawn from zolpidem, and especially in the group where withdrawal lasted for 96 hours (Fig. 4). In our previous studies we also found functional uncoupling of allosteric linkage between GABA and benzodiazepine binding sites already after two-day zolpidem treatment (Vlainic et al. 2010, 2012a) whereas in the study of Primus and colleagues (1996) decline in the allosteric coupling at recombinant GABA_A receptors produced by 1 micromolar zolpidem concentration is reported. Allosteric uncoupling of binding sites is hypothesized as one possible explanation for the decrease of benzodiazepine action at GABA_AR complex (for review see Bateson 2002 and Vinkers and Olivier 2012). There are numerous studies indicating such changes after chronic benzodiazepine treatment (Roca et al. 1990, Klein et al. 1994, Wong et al. 1994, Itier et al. 1996, Primus et al. 1996, Ali and Olsen 2001, Pericic et al. 2007, Svob Strac et al. 2008,

Vlainic et al. 2010, 2012b). As a possible mechanism involved in the functional uncoupling of binding sites at GABA_ARs, Ali and Olsen (2001) proposed changes in conformation of the receptor. Namely, they assumed that chronic benzodiazepine treatment favors a specific receptor conformation which is a substrate for internalization and uncoupling, though the internalization of surface receptors was not observed in other studies (Primus et al. 1996, Jazvinscak Jembrek et al. 2008). Moreover, uncoupling could not be prevented at GABA_ARs expressed in cortical neurons even following lyses of internal vesicles (Gravielle et al. 2005). It has been suggested that the uncoupling occurs in several steps where an initial internalization of receptors is followed by activation of signaling pathway(s) that might lead to selective changes in receptor subunit assembly (Gutierrez et al. 2014). This hypothesis is at least partially suspect since functional uncoupling of GABA and benzodiazepine binding sites following prolonged treatment with benzodiazepines (Klein et al. 1994, 1995, Wong et al. 1994, Primus et al. 1996, Pericic et al. 2007, Svob Strac et al. 2008) or zolpidem (Vlainic et al. 2010, 2012a) was detected with recombinant GABA_ARs with a specific subunit composition where a subunit switch could not occur. Therefore, we hypothesize that uncoupling at recombinant stably transfected GABA_ARs could involve either posttranslational modifications of GABA_AR proteins or changes in subunit stoichiometry. Internalization of receptors in intracellular vesicles where benzodiazepine but not GABA binds to its site could be presumably also excluded, since several studies showed that following chronic drug treatment receptors remain at the cell surface (Primus et al. 1996, Pericic et al. 2007, Jazvinscak Jembrek et al. 2008). Another proposed mechanism is the alteration of the receptor itself through different phosphorylation status which can influence channel openings and/or receptor trafficking. Namely, Lilly and colleagues (2003) found cAMP-dependent protein kinase A (PKA) activity dysfunction following chronic flurazepam treatment. Moreover, Gutierrez and colleagues (2014) in their experiments detected blocking of uncoupling induced by inhibitors of protein kinases A and C, indicating that the uncoupling is mediated by the activation of a phosphorylation cascade.

Dissociation constant (K_d) is the equilibrium constant for the dissociation of a complex into its components. Since the dissociation constant for a particular

ligand-receptor interaction is affected by different conditions, one might assume that zolpidem treatment and its withdrawal could change the strength of intermolecular interactions which holds a particular ligand-receptor complex together. However, in our experiments there was no significant change regarding zolpidem treated and withdrawal groups although we observed a slight increase in the affinity of the GABA_AR (Table II). It has been proposed that such, even small alterations in the affinity of GABA_AR might influence the modulation of ³⁶Cl⁻ efflux, e.g. by decreasing GABA-stimulated chloride influx (Toki et al. 1996).

CONCLUSIONS

This study showed that chronic zolpidem treatment produces no changes in either the number of benzodiazepine binding sites or in the expression of GABA_AR subunits mRNA. In addition, our results demonstrate that zolpidem withdrawal has differing effects depending on the withdrawal duration. Namely, 24, 48 and 72 withdrawal periods have no effects on the number or the expression of mRNA, while the withdrawal period of 96 hours produced an upregulation of [³H]flunitrazepam binding sites along with an enhancement of α 1 and γ 2S mRNAs suggesting an increased synthesis of receptor proteins. Simultaneously, prolonged zolpidem treatment as well as its withdrawal produced an uncoupling of GABA and benzodiazepine binding sites. This study gives an insight into the potential molecular and cellular mechanisms underlying adaptive changes of GABA_AR function following chronic usage of subunit specific GABAergic drugs and one might hypothesize that the observed molecular changes could be linked to zolpidem tolerance and dependence produced upon its prolonged treatment.

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