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Enhancement of benzodiazepine binding sites following chronic treatment with flumazenil

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Abstract

The aim of this study was to improve our knowledge of the mechanisms leading to adaptive changes in γ-aminobutyric acid$_A$ (GABA$_A$) receptors following chronic drug treatment. Exposure (48 h) of human embryonic kidney (HEK 293) cells stably expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors to the antagonist of benzodiazepine binding sites, flumazenil (5 μM), enhanced the maximum number (B_{max}) and the equilibrium dissociation constant (K_d) of [^{3}H]flunitrazepam binding sites. The flumazenil-induced enhancement in B_{max} was potentiated by GABA (50 μM) and reduced by the GABA$_A$ receptor antagonist, bicuculline (100 μM). Flumazenil-induced enhancement in K_d was affected by neither of these treatments. GABA (50 μM) enhanced the density of [^{3}H]flunitrazepam binding sites, and this enhancement was greater in the presence of diazepam (1 μM). The results suggest that chronic flumazenil treatment up-regulates in a bicuculline-sensitive manner benzodiazepine binding sites at stably expressed GABA$_A$ receptors.

Keywords: GABA$_A$ receptor; HEK 293 cell; Flumazenil; Diazepam; GABA (γ-aminobutyric acid); [^{3}H]flunitrazepam binding

1. Introduction

γ-Aminobutyric acid$_A$ (GABA$_A$) receptors mediate fast inhibitory neurotransmission in the mammalian central nervous system. These receptors belong to a superfamily of ligand-gated ion-channels composed of various polypeptide subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ∈, π, θ, ρ_{1-3}) that are encoded by different genes (Mehta and Ticku, 1999; Sieghart et al., 1999; Whiting, 1999; Rudolph et al., 2001; Korpi et al., 2002). However, most GABA$_A$ receptors are comprised of α, β, and γ subunits (Barnard et al., 1998; Farrar et al., 1999), and the most abundant subunit combination found in the brain is $\alpha_1\beta_2\gamma_2$ (McKernan and Whiting, 1996).

GABA$_A$ receptors possess binding sites for a variety of drugs, such as anxiolytics, anticonvulsants, general anesthetics, barbiturates, ethanol, and neurosteroids, which are known to elicit at least some of their pharmacological effects via GABA$_A$ receptors (Johnston, 1996; Mehta and Ticku, 1999; Korpi et al., 2002). The functional and pharmacological properties of GABA$_A$ receptors, including the potency and efficacy of the neurotransmitter itself, depend on their subunit composition (Korpi et al., 2002).

Prolonged occupancy of GABA$_A$ receptors by agonists and drugs facilitating the action of GABA leads to regulatory changes generally resulting in the down-regulation of receptor levels and function. Thus, prolonged treatment with benzodiazepines, barbiturates, and steroids (Gallager et al., 1984; Roca et al., 1990; Friedman et al., 1996) induces an allosteric uncoupling of GABA and benzodiazepine binding sites, characterized by a decrease in the GABA enhancement of benzodiazepine binding. It has been suggested that this phenomenon may be related to the development of tolerance and physical dependence, which appear in animals and humans following prolonged treatment with these drugs

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(Gallager et al., 1984; Roca et al., 1990). Chronic benzodiazepine antagonist treatment appears to affect $GABA_A$ receptor number and/or function oppositely from chronic agonist treatment (Miller et al., 1989; Urbancic and Marczynski, 1989; Kulkarni, 1990).

Unlike native $GABA_A$ receptors, which are heterogenous (Mehta and Ticku, 1999; Sieghart et al., 1999; Whiting, 1999; Rudolph et al., 2001; Korpi et al., 2002), recombinant receptors transiently or stably expressed in transfected cells have well-defined subunit composition. Although this offers some advantages over the studies on the native receptors, the reports related to prolonged exposure of these cells to drugs, such as benzodiazepines, are not numerous (Wong et al., 1992, 1994; Klein et al., 1995; Primus et al., 1996; Ali and Olsen, 2001; Peričić et al., 2003). Even less frequent are the studies, which report the effects of prolonged exposure to flumazenil, the antagonist of benzodiazepine binding sites (Wong et al., 1994; Klein et al., 1995; Primus et al., 1996). Such studies might be important since flumazenil is not only being used acutely to reverse the adverse effects of over sedation with benzodiazepines, but it is also being considered for treatment of hepatic encephalopathy patients (Als-Nielsen et al., 2001; Goulenok et al., 2002; Dursun et al., 2003). Therefore, in this study, stably transfected HEK 293 cells (Besnard et al., 1997) were used as a model to study the effects of prolonged flumazenil exposure on the recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors, the most common subunit combination of native $GABA_A$ receptors in the mammalian central nervous system (CNS; McKernan and Whiting, 1996), with aim to improve our knowledge on the mechanisms that underlie adaptive changes in $GABA_A$ receptors following their prolonged exposure to drugs.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney (HEK) 293 cell line expressing the $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptor was kindly donated by Dr. David Graham (Sanofi-Synthélabo Research, France). The cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin in 75 cm^2 Falcon flasks according to standard cell culture techniques.

2.2. Drugs

Flumazenil (Ro 15-1788) was from F. Hoffmann-La Roche (Basel, Switzerland). Diazepam, bicuculline, and GABA were from Sigma (St. Louis, MO). [^{3}H]flunitrazepam (specific activity 85 Ci/ mmol) was purchased from Du Pont, NEN. Culture medium, antibiotics, and fetal bovine serum were from Sigma.

2.3. Drug treatment

Three days prior to exposure to drugs, the cells were transferred to new flasks and grown in the above medium. Three days after seeding, the medium was removed and replaced with fresh medium containing drugs or vehicles. Flumazenil (final concentration 1 or 5 μM), diazepam (final concentration 1 μM), GABA (final concentration 50 μM), and bicuculline (final concentration 100 μM) were used. Flumazenil was dissolved in ethanol at the 1 mM concentration, GABA was dissolved in distilled water, diazepam in 0.1 N HCl, and bicuculline in warm 0.1 N HCl. Control cells were treated with the corresponding vehicles. The cells were treated with drugs for 48 h, except in the experiment with 50 μM GABA in combination with 1 μM diazepam, in which the cells were exposed to drugs for 96 h. In that case, the drugs were replaced after 2 days in culture.

2.4. Preparation of the membranes

Membranes from stably transfected HEK 293 cells were prepared mainly as described by Fuchs et al. (1995). The cells were washed, harvested by scraping into phosphate-buffered saline, 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 (up and down) in a glass/Teflon homogenizer at 1250 rpm, and then centrifuged at 200 000×g for 20 min. The pellet was resuspended, centrifuged at 200 000×g for 20 min two more times, resuspended again, and stored at −20 °C. On the day of assay, the suspension was centrifuged once more at 200 000×g for 20 min and used for binding studies.

2.5. [^{3}H]Flunitrazepam binding assay

Aliquots of the cell membrane preparation (~75 μg protein) were incubated in 50 mM Tris–citrate buffer (pH 7.4) containing 150 mM NaCl at 4 °C for 90 min with [^{3}H]flunitrazepam (0.2–16 nM). The total assay volume was 0.5 ml. Nonspecific [^{3}H]flunitrazepam binding, defined in the presence of 100 μM diazepam, was less than 5% of the total binding (at the concentration of [^{3}H]flunitrazepam 1 nM). Radioactivity bound to membranes was determined after rapid filtration on Whatman GF/C filters.

2.6. Protein determination

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

2.7. Data analysis

The analysis of binding data was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA. The values K_d and B_{max}

were obtained by nonlinear regression using the equation for hyperbola (one binding site): $Y=B_{max}*X/(K_d+X)$, where B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding.

Results are expressed as the mean±S.E.M. or as the percent of control. Statistical analysis of results was by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. Student's *t*-test was also used where appropriate. *P*-values of <0.05 were considered significant.

3. Results

3.1. The effect of chronic flumazenil treatment on [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of GABA$_A$ receptors

As shown in Figs. 1 and 2, [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of GABA$_A$ receptors showed differences

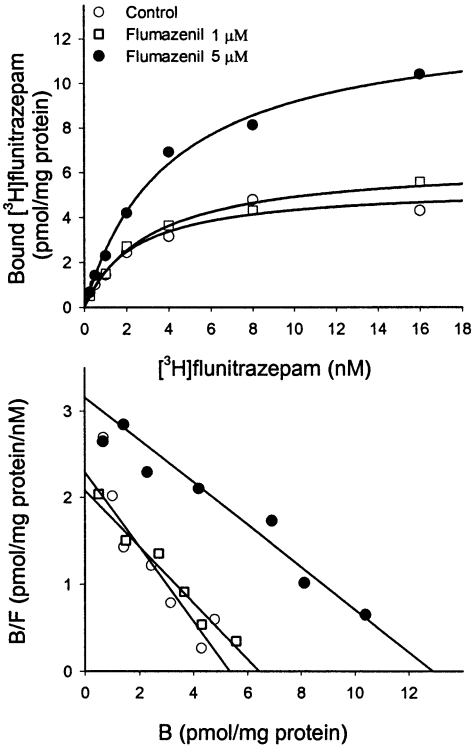

Fig. 1. Saturation isotherms and Scatchard plot of [^{3}H]flunitrazepam binding to membranes from HEK 293 cells treated with flumazenil. HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of GABA$_A$ receptors were treated with 1 or 5 μM of flumazenil for 48 h. Cell membranes from flumazenil-treated and control (vehicle-treated) cells were prepared as described in Materials and methods and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM). Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. The figure demonstrates one typical experiment. B_{max} and K_d values were obtained by nonlinear regression using GraphPad Prism version 4.00. Scatchard line was obtained by connecting the *X* and *Y* axis intercepts that accurately reflect the K_d and B_{max} values as determined by nonlinear regression.

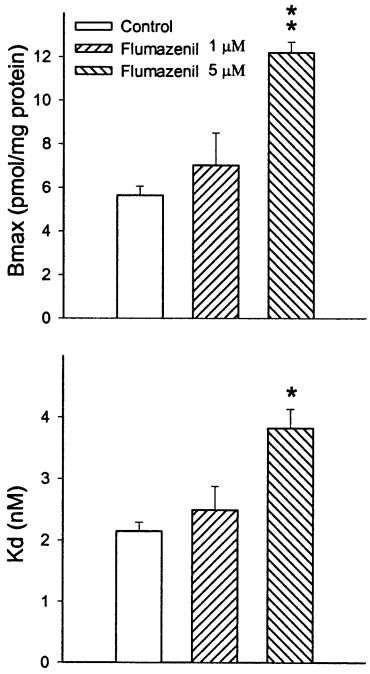

Fig. 2. The effect of chronic flumazenil treatment on [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of GABA$_A$ receptors. The cells were treated with 1 or 5 μM of flumazenil for 48 h. Results are expressed as means±S.E.M. Treated samples were always compared with matched controls that were cultured and assayed the same day as the treated samples. Data for control groups are derived from six, and for each flumazenil-treated group from three separate experiments performed in duplicate. *P<0.01, **P<0.001 versus control (ANOVA followed by the Newman–Keuls test).

between the three treated groups (control, flumazenil 1 μM, flumazenil 5 μM). Newman–Keuls test following one-way ANOVA [$F(2,9)=19.53$; $P<0.0005$] indicated that the B_{max} value was enhanced ($P<0.001$ vs. control vehicle-treated group) by exposure of cells to 5 but not to 1 μM of flumazenil. ANOVA also revealed significant differences in the affinity of these binding sites [$F(2,9)=12.29$; $P<0.003$], and post hoc analysis by Newman–Keuls test indicated that the K_d value was enhanced ($P<0.01$) by exposure of cells to 5 but not to 1 μM of flumazenil.

Pretreatment of HEK 293 cells with flumazenil (5 μM) for 30 min failed to affect the maximum number, but it enhanced the K_d value of [^{3}H]flunitrazepam binding sites [control: 2.49±0.06 nM (n=5); Flumazenil: 3.27±0.18 nM (n=5); $P<0.01$, Student's *t*-test].

3.2. The effect of bicuculline on flumazenil-induced up-regulation of [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of GABA$_A$ receptors

To test whether GABA binding sites were involved in the enhancement of [^{3}H]flunitrazepam binding following treatment with 5 μM flumazenil for 48 h, we treated one group of cells simultaneously with flumazenil and bicuculline, the competitive antagonist of GABA binding sites. As shown in

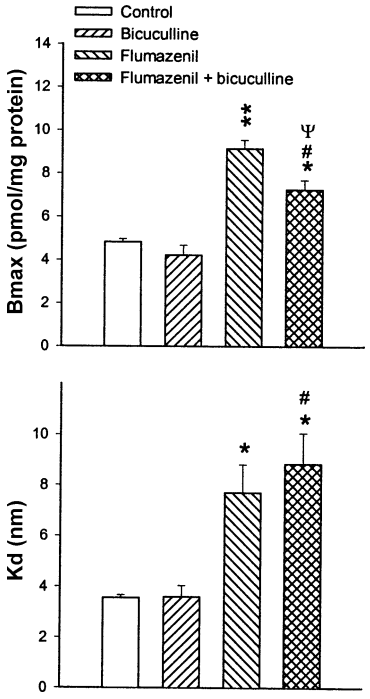

Fig. 3. The ability of bicuculline to reduce chronic flumazenil-induced enhancement of [^{3}H]flunitrazepam binding sites in the membranes of HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors. HEK 293 cells were treated for 48 h with 5 μM flumazenil or 100 μM bicuculline, with a combination of both drugs, or with their vehicles. Results are expressed as means±S.E.M from three separate experiments performed in duplicate. *P<0.05 or P<0.01 versus control, **P<0.001 versus control; $^{\Psi}P$<0.01 versus flumazenil; $^{\#}P$<0.01 versus bicuculline-treated group (ANOVA followed by the Newman–Keuls test).

Fig. 3 and indicated by one-way ANOVA [$F(3,8)$=34.71; P<0.0001], the maximum number of [^{3}H]flunitrazepam binding sites between the four treated groups (control, flumazenil, bicuculline, flumazenil+bicuculline) was different. Flumazenil (5 μM) again produced a great enhancement of [^{3}H]flunitrazepam binding sites. Bicuculline (100 μM) decreased the effect of flumazenil (P<0.01), but bicuculline alone, in accordance with our previous study (Peričić et al., 2003), had no effect on the control expression levels.

As indicated by one-way ANOVA [$F(3,8)$=10.31; P<0.004], the four treated groups of cells also showed differences in the affinity of [^{3}H]flunitrazepam binding sites. Flumazenil enhanced significantly (P<0.05) the K_d value of [^{3}H]flunitrazepam binding, and this enhancement was still present when cells were cotreated with bicuculline.

3.3. The effect of GABA on flumazenil-induced up-regulation of [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors

To test whether previously observed up-regulation of [^{3}H]flunitrazepam binding sites following chronic treatment with GABA (Peričić et al., 2003) will affect flumazenil-induced up-regulation of the same binding sites, we treated one group of cells with flumazenil (5 μM) and GABA (50 μM) for 48 h.

One-way ANOVA [$F(3,8)$=37.76] indicated significant differences (P<0.0001) between the four treated groups (control, GABA, flumazenil, flumazenil+GABA). Both GABA and flumazenil produced enhancements (91% and 72% compared to control) in B_{max} values (P<0.05, Newman–Keuls test). The greatest enhancement (330%), which was significantly different not only from control (P<0.001), but also from cells treated either with GABA or flumazenil alone (P<0.001), was observed in membranes obtained from cells subjected to combined GABA+flumazenil treatment (Fig. 4A).

The flumazenil-induced changes in K_d value were not affected by GABA (data not shown).

3.4. The effect of combined GABA and diazepam treatment on [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors

As shown in Fig. 4B, the enhanced density of [^{3}H]flunitrazepam binding sites obtained following prolonged treat-

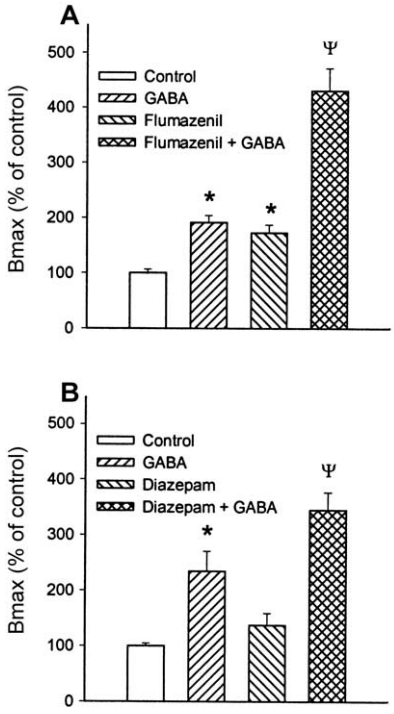

Fig. 4. The effect of combined chronic GABA and flumazenil or GABA and diazepam treatment on the maximum number of [^{3}H]flunitrazepam binding sites on the membranes of HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors. HEK 293 cells were treated (A) for 48 h with 5 μM flumazenil or 50 μM GABA, the combination of both drugs, or with their vehicles; (B) for 96 h with 1 μM diazepam or 50 μM GABA, the combination of both drugs, or with their vehicles. Results are expressed as percent of control±S.E.M from three separate experiments performed in duplicate. (A) *P<0.05 versus control; $^{\Psi}P$<0.001 versus all other groups; (B) *P<0.05 versus control; $^{\Psi}P$<0.05 versus GABA, P<0.01 versus diazepam, P<0.001 versus control (ANOVA followed by the Newman–Keuls test).

ment with GABA (50 μM, 96 h) was further increased when HEK 293 cells expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors were cotreated with 1 μM diazepam (GABA+diazepam: $P<0.05$ vs. GABA; $P<0.01$ vs. diazepam; $P<0.001$ vs. control; Newman–Keuls test), although diazepam alone failed to affect the B_{max} value. Four treated groups failed to show differences in the affinity of benzodiazepine binding sites [$F(3,8)=0.77$].

4. Discussion

The present study demonstrated that prolonged exposure of HEK 293 cells stably expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ subunits of the rat $GABA_A$ receptors to flumazenil enhances the maximum number and reduces the affinity of [^{3}H]flunitrazepam binding sites. Chronic flumazenil-induced up-regulation of benzodiazepine binding sites was potentiated by GABA and diminished by bicuculline, competitive antagonist of GABA binding sites. GABA also up-regulated benzodiazepine binding sites, and this effect was enhanced in the presence of diazepam.

The up-regulation of [^{3}H]flunitrazepam binding sites following chronic flumazenil treatment has already been obtained with native receptors. Miller et al. (1990) observed an increase of [^{3}H]flunitrazepam binding sites in cultured cortical neurons following treatment for 10 days with either flumazenil (1 μM) or the inverse agonist FG 7142. Moreover, both of these drugs lead to increases in GABA-dependent chloride uptake. The up-regulation of binding sites for benzodiazepines and convulsants, as well as the enhancement of $GABA_A$ receptor function, as determined by chloride uptake into cortical synaptoneurosomes, was also observed when mice were subjected to chronic flumazenil administration via implanted osmotic pumps (Miller et al., 1989). The chronic flumazenil treatment and its withdrawal also increased the specific [^{3}H]flunitrazepam binding in the cerebral cortex of rat (Medina et al., 1983; Urbancic and Marczynski, 1989; Kulkarni, 1990). However, previous results with recombinant receptors were at variance with aforementioned studies. Thus, Wong et al. (1994) failed to observe a change in the density of benzodiazepine binding to WSS-1 cells following 48 h exposure to different concentrations of flumazenil. In our study, chronic flumazenil enhanced the maximum number of [^{3}H]flunitrazepam binding sites, and this change was accompanied by a decrease in their affinity. Change in the affinity but not in receptor number was seen already after a short exposure (30 min) of cells to flumazenil, suggesting that even the extensive washing did not remove the entire drug from cell membranes. Hence, the change in the affinity of receptors does not appear to be the consequence of the chronic drug treatment.

Treatment with diazepam, consistent with our previous study (Peričić et al., 2003) and the results of other in vivo (Gallager et al., 1984) and in vitro studies (Hu and Ticku, 1994; Wong et al., 1994; Primus et al., 1996), failed to modify the kinetic parameters (B_{max} and K_d) of [^{3}H] flunitrazepam binding. It has been shown that diazepam in the presence of low concentrations of GABA produces a biphasic potentiation at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors via two distinct mechanisms. Only high-affinity potentiation (nM component) is sensitive to flumazenil (Walters et al., 2000), suggesting that this benzodiazepine antagonist produces its effects via high-affinity sites.

The enhanced B_{max}, but not the K_d, of [^{3}H] flunitrazepam binding sites, observed after chronic flumazenil treatment, was inhibited by bicuculline, suggesting that GABA recognition site is important for the effect of flumazenil. Our data demonstrating that cotreatment with GABA (50 μM) potentiated flumazenil-induced enhancement of [^{3}H] flunitrazepam binding sites might suggest that the two drugs act by two different mechanisms that are mutually stimulative. This was unexpected, since it is well known that GABA enhances the affinity for agonists, but not for the antagonists of benzodiazepine binding sites (Möhler and Richards, 1981). As shown in Fig. 4B, the prolonged exposure to 50 μM GABA in combination with diazepam also produced a greater enhancement in B_{max} than GABA alone. Hence, perhaps the effects of flumazenil might be explained by the fact that this drug might, as previously reported (Skerritt and MacDonald, 1983; Buldakova and Weiss, 1997; Weiss et al., 2002), behave like a partial agonist at the $GABA_A$ receptor.

Up-regulation of binding sites for benzodiazepines and convulsants following chronic treatment with GABA has recently been described (Peričić et al., 2003). In the mentioned study, the GABA-induced enhancement in B_{max} of [^{3}H] flunitrazepam binding sites was reduced by the GABA receptor antagonist, bicuculline, and by cycloheximide, a protein synthesis inhibitor, suggesting an increased synthesis rather than a decreased degradation of the receptor protein.

Further studies should demonstrate whether the enhanced expression of receptor number following chronic flumazenil is the result of an increased synthesis or a decreased degradation of receptor protein. Enhanced rates of receptor incorporation into membrane would have the same results. A general trophic effect of flumazenil in stably transfected HEK 293 cells could presumably be excluded, since total cellular proteins in flumazenil-treated cells were not increased (data not shown). Regardless of the mechanism involved, this study suggests that chronic treatment with the antagonist, and not only with agonists of benzodiazepine binding sites, is able to induce adaptive changes in $GABA_A$ receptors in an in vitro system.

Although one might argue that with regard to the mechanisms controlling the expression of receptors (recombinant versus native), and with regard to the absence or presence of endogenous GABA and a normal neuronal environment, the transfected HEK 293 cells were an artificial system, our results with flumazenil obtained

with the recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors are mainly in agreement with the data obtained on the brain, where the native $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptors predominates (McKernan and Whiting, 1996). This and a relatively high density of binding sites (Peričić et al., 1998, 2001, 2003) suggest that stably transfected HEK 293 cells used in this study appear to be a suitable model for studying mechanisms that underlie adaptive changes in $GABA_A$ receptors following their prolonged exposure to drugs. Moreover, it has recently been demonstrated that HEK 293 cells have an unexpected relationship to neurons (Shaw et al., 2002).

In conclusion, prolonged treatment of cells expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors with benzodiazepine antagonist flumazenil enhances the maximum number of [^{3}H]flunitrazepam binding sites. Chronic flumazenil-induced changes in the receptor density are potentiated by GABA and are sensitive to bicuculline. Further studies are needed to elucidate these phenomena.

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References

Ali, N.J., Olsen, R.W., 2001. Chronic benzodiazepine treatment of cells expressing recombinant $GABA_A$ receptors uncouples allosteric binding: studies on possible mechanisms. J. Neurochem. 79, 1100–1108.

Als-Nielsen, B., Kjaergard, L.L., Gluud, C., 2001. Benzodiazepine receptor antagonists for acute and chronic hepatic encephalopathy. Cochrane Database Syst. Rev. 4, CD002798.

Barnard, E.A., Skolnick, P., Olsen, R.W., Möhler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N., Langer, Z., 1998. International union of pharmacology: XV. Subtypes of γ-Aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. Pharmacol. Rev. 50, 291–313.

Besnard, F., Even, Y., Itier, V., Granger, P., Partiseti, M., Avenet, P., Depoortere, H., Graham, D., 1997. Development of stable cell lines expressing different subtypes of $GABA_A$ receptors. J. Recept. Signal Transduct. Res. 17, 99–113.

Buldakova, S., Weiss, M., 1997. Electrophysiological evidence for agonist properties of flumazenil, a benzodiazepine receptor antagonist, in rat hippocampus slices. J. Neurol. Sci. 149, 121–126.

Dursun, M., Caliskan, M., Canoruc, F., Aluclu, U., Canoruc, N., Tuzcu, A., Yilmaz, S., Isikdogan, A., Ertem, M., 2003. The efficacy of flumazenil in subclinical to mild hepatic encephalopathic ambulatory patients—a prospective, randomized, double-blind, placebo-controlled study. Swiss Med. Wkly. 133, 118–123.

Farrar, S.J., Whiting, P.J., Bonnert, T.P., McKernan, R.M., 1999. Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J. Biol. Chem. 274, 10100–10104.

Friedman, L.K., Gibbs, T.T., Farb, D.H., 1996. γ-Aminobutyric acid A receptor regulation: heterologous uncoupling of modulatory site interactions induced by chronic steroid, barbiturate, benzodiazepine, or GABA treatment in culture. Brain Res. 707, 100–109.

Fuchs, K., Zezula, J., Slany, A., Sieghart, W., 1995. Endogenous [^{3}H]flunitrazepam binding in human embryonic kidney cell. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 289, 87–95.

Gallager, D.W., Lakoski, J.M., Gonsalves, S.F., Rauch, S.L., 1984. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. Nature 308, 74–77.

Goulenok, C., Bernard, B., Cadranel, J.F., Thabut, D., Di Martino, V., Opolon, P., Poynard, T., 2002. Flumazenil vs. placebo in hepatic encephalopathy in patients with cirrhosis: a metaanalysis. Aliment. Pharmacol. Ther. 16, 361–372.

Hu, X.J., Ticku, M.K., 1994. Chronic benzodiazepine agonist treatment reduces functional uncoupling of the γ-aminobutyric acid–benzodiazepine receptor ionophore complex in cortical neurons. Mol. Pharmacol. 45, 618–625.

Johnston, G.A.R., 1996. $GABA_A$ receptor pharmacology. Pharmacol. Ther. 69, 173–198.

Klein, R.L., Mascia, M.P., Harkness, P.C., Hadingham, K.L., Whiting, P.J., Harris, R.A., 1995. 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. 274, 1484–1492.

Korpi, E.R., Grunder, G., Lüddens, H., 2002. Drug interactions at GABA (A) receptors. Prog. Neurobiol. 67, 113–159.

Kulkarni, S.K, Ticku, M.K., 1990. Chronic benzodiazepine antagonist treatment and its withdrawal upregulates components of GABA–benzodiazepine receptor ionophore complex in cerebral cortex of rat. Brain Res. 519, 6–11.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

McKernan, R.M., Whiting, P.J., 1996. Which GABA (A)-receptor subtypes really occur in the brain. Trends Neurosci. 19, 139–143.

Medina, J.H., Novas, M.L., De Robertis, E., 1983. Chronic Ro 15-1788 treatment increases the number of benzodiazepine receptors in rat cerebral cortex and hippocampus. Eur. J. Pharmacol. 90, 125–128.

Mehta, A.K., Ticku, M.K., 1999. An update on $GABA_A$ receptors. Brain Res. Brain Res. Rev. 29, 196–217.

Miller, L.G., Greenblatt, D.J., Roy, R.B., Gaver, A., Lopez, F., Shader, R.I., 1989. Chronic benzodiazepine administration: III. Upregulation of gamma-aminobutyric acid A receptor binding and function associated with chronic benzodiazepine antagonist administration. J. Pharmacol. Exp. Ther. 248, 1096–1101.

Miller, L.G., Heller, J., Lumpkin, M., Weill, C.L., Greenblatt, D.J., Shader, R.I., 1990. Augmentation of $GABA_A$ receptor function by chronic exposure to GABA-neutral and GABA-negative benzodiazepine ligands in cultured cortical neurons. Biochem. Pharmacol. 40, 1337–1344.

Möhler, H., Richards, J.G., 1981. Agonist and antagonist benzodiazepine receptor interaction in vitro. Nature 294, 763–765.

Peričić, D., Mirković, K., Jazvinšćak, M., Besnard, F., 1998. [^{3}H]t-butylbicycloorthobenzoate binding to recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor. Eur. J. Pharmacol. 360, 99–104.

Peričić, D., Jazvinšćak, M., Mirković, K., 2001. [^{3}H]Flunitrazepam binding to recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors stably expressed in HEK 293 cells. Biomed. Pharmacother. 55, 221–228.

Peričić, D., Švob Štrac, D., Jazvinšćak Jembrek, M., Rajčan, I., 2003. Prolonged exposure to γ-aminobutyric acid up-regulates stably expressed recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. Eur. J. Pharmacol. 482, 117–125.

Primus, R.J., Yu, J., Xu, J., Hartnett, C., Meyyappan, M., Kostas, C., Ramabhadran, T.V., Gallager, D.W., 1996. 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. 276, 882–890.

Roca, D.J., Schiller, G.D., Friedman, L., Rozenberg, I., Gibbs, T.T., Farb, D.H., 1990. γ-Aminobutyric $acid_A$ receptor regulation in culture: altered allosteric interactions following prolonged exposure to benzodiazepines, barbiturates, and methylxanthines. Mol. Pharmacol. 37, 710–719.

Rudolph, U., Crestani, F., Möhler, H., 2001. GABA (A) receptor subtypes: dissecting their pharmacological functions. Trends Pharmacol. Sci. 22, 402–403.

Shaw, G., Morse, S., Ararat, M., Graham, F.L., 2002. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. FASEB J. 16, 869–871.

Sieghart, W., Fuchs, K., Tretter, V., Ebert, V., Jechlinger, M., Hoger, H., Adamiker, D., 1999. Structure and subunit composition of $GABA_A$ receptors. Neurochem. Int. 34, 379–385.

Skerritt, J.H., MacDonald, R.L., 1983. Benzodiazepine Ro 15-1788: electrophysiological evidence for partial agonist activity. Neurosci. Lett. 43, 321–326.

Urbancic, M., Marczynski, T.J., 1989. Chronic exposure to Ro 15-1788: differential effect on flunitrazepam binding to cortex and hippocampus. Eur. J. Pharmacol. 171, 1–7.

Walters, R.J., Hadley, S.H., Morris, K.D.W., Amin, J., 2000. Benzodiazepines act on $GABA_A$ receptors via two distinct and separable mechanisms. Nat. Neurosci. 3, 1274–1281.

Weiss, M., Tikhonov, D., Buldakova, S., 2002. Effect of flumazenil on $GABA_A$ receptors in isolated rat hippocampal neurons. Neurochem. Res. 27, 1605–1612.

Whiting, P.J., 1999. The GABA-A receptor gene family: new targets for therapeutic intervention. Neurochem. Int. 34, 387–390.

Wong, G., Yoshitatsu, S., Skolnick, P., 1992. Stable expression of type I γ-aminobutyric$acid_A$/benzodiazepine receptors in a transfected cell line. Mol. Pharmacol. 42, 996–1003.

Wong, G., Lyon, T., Skolnick, P., 1994. Chronic exposure to benzodiazepine receptor ligands uncouples the γ-aminobutyric acid type A receptor in WSS-1 cells. Mol. Pharmacol. 46, 1056–1062.