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Low tolerance and dependence liabilities of etizolam: Molecular, functional, and pharmacological correlates

Enrico Sanna [a,b,*], Fabio Busonero [a,b], Giuseppe Talani [a,b], Maria Cristina Mostallino [c], Maria Luisa Mura [a,b], Maria Giuseppina Pisu [c], Elisabetta Maciocco [c], Mariangela Serra [a,b,c], Giovanni Biggio [a,b,c]

[a] *Department of Experimental Biology, Section of Neuroscience, University of Cagliari, Cagliari, Italy*
[b] *Center of Excellence for the "Neurobiology of Dependence", University of Cagliari, Cagliari, Italy*
[c] *Consiglio Nazionale delle Ricerche (C.N.R.), Institute of Neuroscience, Cagliari, Italy*

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Abstract

The effects of prolonged exposure to and subsequent withdrawal of the thienotriazolobenzodiazepine etizolam on γ-aminobutyric acid (GABA) type A receptor gene expression and function were compared with those of the benzodiazepine lorazepam. Exposure of rat hippocampal neurons in culture to 10 μM etizolam for 5 days reduced the amounts of α5 and γ2S receptor subunit mRNAs, whereas etizolam withdrawal was associated with a persistent reduction in γ2S mRNA and an increase in α2 and α3 mRNAs. Neither chronic exposure to nor withdrawal of etizolam affected the acute modulatory effects of etizolam or lorazepam on GABA-evoked Cl^- current. Treatment with 10 μM lorazepam for 5 days reduced the amounts of α1 and γ2S subunit mRNAs and increased that of α3 mRNA, whereas lorazepam withdrawal was associated with persistence of the changes in α3 and γ2S mRNAs and an increase in α2 and α4 mRNAs. Parallel changes in the abundance of α1 and α4 subunit proteins induced by chronic exposure to and withdrawal of lorazepam, but not etizolam, were detected by immunocytofluorescence analysis. Chronic lorazepam treatment resulted in a reversible reduction in the modulatory efficacy of this drug and conferred on flumazenil the ability to potentiate GABA-evoked Cl^- current. The anticonvulsant action of etizolam was not altered in mice chronically treated with this drug, whereas lorazepam-treated animals became tolerant to the acute anticonvulsant effect of this benzodiazepine. These data suggest that etizolam is endowed with a reduced liability to induce tolerance and dependence compared with classical benzodiazepines.
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Keywords: Etizolam; Benzodiazepines; $GABA_A$ receptor; Tolerance; Dependence; Gene expression

1. Introduction

Although benzodiazepines are effective anxiolytic, anti-epileptic, and hypnotic drugs, the development of tolerance to certain of their effects greatly limits the long-term clinical use of these compounds (Bateson, 2002; File, 1985; Haigh and Feely, 1988; Stewart and Westra, 2002). Chronic administration of benzodiazepines is also associated with the development of physical dependence (Ryan and Boisse, 1983; Schweizer and Rickels, 1998). The neurobiological and molecular mechanisms that underlie such long-term effects of benzodiazepines are, for the most part, unknown. Tolerance and dependence appear to correlate, however, with pharmacodynamic processes rather than with pharmacokinetic characteristics of benzodiazepines (File, 1985; Gallager et al., 1984, 1985, 1991).

The best characterized neurobiological mechanisms thought to contribute to benzodiazepine tolerance are down-regulation of the type A receptor for γ-aminobutyric acid (GABA) and the associated benzodiazepine receptor

* Corresponding author. Department of Experimental Biology, Section of Neuroscience, University of Cagliari, Cittadella Universitaria, SS 554, km 4500, 09042 Monserrato (CA), Italy. Tel.: +39 070 675 4139; fax: +39 070 675 4166.
E-mail address: esanna@unica.it (E. Sanna).

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(Itier et al., 1996; Zeng and Tietz, 1999) as well as functional uncoupling of this $GABA_A$-benzodiazepine receptor complex (Hu and Ticku, 1994). The change in $GABA_A$ receptor function is related to alterations in receptor subunit composition (Follesa et al., 2001; Impagnatiello et al., 1996; Kang and Miller, 1991; Li et al., 2000; Tietz et al., 1999). Changes in the abundance of α1, β2, and β3 subunits of the $GABA_A$ receptor have been suggested to constitute a minimal requirement for the development of tolerance to the anticonvulsant effect of benzodiazepines. The amounts of messenger RNAs (mRNAs) for the α1 and β2 subunits were thus found to be reduced and that of the β3 subunit mRNA to be increased in the hippocampus of flurazepam-tolerant rats (Tietz et al., 1999).

The thienotriazolobenzodiazepine derivative etizolam (Nakanishi et al., 1972; Tsumagari et al., 1978) possesses anxiolytic but not sedative properties and exhibits a lower intrinsic activity at α1 subunit-containing $GABA_A$ receptors than do classical benzodiazepines. Both the potency and efficacy of etizolam for allosteric potentiation of GABA-evoked Cl^- current mediated by recombinant α1β2γ2S receptors are thus lower than those of the benzodiazepine alprazolam (Sanna et al., 1999). These properties, together with pharmacokinetics indicative of a short-acting drug (Fracasso et al., 1991), suggest that etizolam might have a reduced liability for the development of tolerance and dependence, side effects typically associated with full agonists of the benzodiazepine receptor (Costa, 1998; Woods et al., 1992).

We have now evaluated the effects of prolonged exposure to and subsequent withdrawal of etizolam, in comparison with those of the benzodiazepine lorazepam, on $GABA_A$ receptor gene expression and pharmacological sensitivity in primary cultures of rat hippocampal neurons. In addition, we have tested the pharmacological efficacy of an anticonvulsant dose of etizolam in antagonizing seizure activity elicited by isoniazid, an inhibitor of GABA synthesis (Horton et al., 1979), in mice chronically treated with etizolam, again comparing the effects of this drug with those induced by similar treatment with lorazepam.

2. Materials and methods

2.1. Primary culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from Sprague–Dawley rats on postnatal days 1–3 as described previously (Costa et al., 2000), with minor modifications. Pups were killed by decapitation, and the hippocampus was removed and transferred to a culture dish containing Neurobasal A medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 25 μM glutamate, 0.5 mM glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml). The tissue was chopped with scissors, and the resulting fragments were transferred to a sterile tube and gently dissociated by repeated passage through a Pasteur pipette with an opening of 0.5 mm. The dissociated cells were plated either in 35-mm culture dishes (4×10^6 cells) that had been coated with poly-L-lysine hydrobromide (100 μg/ml; 30–70 kDa) (Sigma) for measurement of $GABA_A$ receptor subunit mRNAs or in multiwell dishes containing 12-mm round glass cover slips coated with poly-L-lysine (6×10^5 cells) for electrophysiological recording or immunocytochemistry. Cells were cultured in a humidified incubator containing 5% CO_2 at 37 °C. Twenty-four hours after plating, fetal bovine serum was replaced with B-27 supplement (Invitrogen), and glutamate was removed from the medium after culture of the cells for 3 days.

2.2. Drug treatment of cultured neurons

After 5 days in culture, cells were exposed continuously for the next 5 days to 10 μM etizolam (gift from Schering, Italy) or 10 μM lorazepam (gift from Wyeth, Italy), with replacement of the culture medium daily. For assessment of the effects of chronic etizolam or lorazepam treatment, the cultured cells were analyzed immediately after drug removal (0 h of withdrawal). In withdrawal experiments, the drug-containing medium was replaced after 5 days with drug-free medium and the cells were incubated for an additional 6 h. Etizolam and lorazepam were dissolved in dimethyl sulfoxide and subsequently diluted to the desired concentration in culture medium. Control neurons were treated with the corresponding concentration of vehicle. All experimental groups were compared with control cells maintained in culture for an identical time.

2.3. Riboprobe preparation

$GABA_A$ receptor subunit cDNAs were prepared as previously described (Follesa et al., 1998) by reverse transcription and the polymerase chain reaction. In brief, cDNA prepared from rat brain (1–10 ng) was subjected to amplification with *Taq* DNA polymerase (2.5 U) (Perkin-Elmer/Cetus, Norwalk, CT) in 100 μl of standard buffer [100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin] containing 1 μM each of specific sense and antisense primers and 200 μM of each deoxynucleoside triphosphate. The primer pairs for the various receptor subunits were designed to include cDNA sequences with the lowest degree of homology among the different subunits (Follesa et al., 1998). The reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) for 30 cycles of 94 °C for 45 s and 60 °C for 1 min, with a final extension at 72 °C for 15 min. The reaction products were separated by agarose gel electrophoresis, visualized by staining with ethidium bromide, excised from the gel, purified, and cloned into the pAMP 1 vector (Invitrogen). The resulting plasmids were introduced into *Escherichia*

coli DH5α and subsequently purified from the bacterial cells, and the cDNA inserts were sequenced with a Sequenase DNA sequencing kit (USB, Cleveland, OH). The determined nucleotide sequences were 100% identical to those previously published. Plasmids containing the cDNA fragments corresponding to the various $GABA_A$ receptor subunits were linearized with restriction enzymes (Follesa et al., 1998) and used as templates for the appropriate RNA polymerase (SP6 or T7) to generate [α-^{32}P]UTP-labeled cRNA probes for RNase protection assays.

2.4. RNA extraction and measurement of $GABA_A$ receptor subunit mRNAs

Total RNA was isolated from cultured hippocampal cells with an RTN kit (Sigma) and was quantified by measurement of absorbance at 260 nm. An RNase protection assay for the semiquantitative measurement of the $GABA_A$ receptor α1 to α5 and γ2S subunit mRNAs was performed as described (Follesa et al., 1998). In brief, 15 μg of total RNA were dissolved in 20 μl of hybridization solution containing 150,000 cpm of ^{32}P-labeled cRNA probe for a specific $GABA_A$ receptor subunit mRNA (6×10^7 to 7×10^7 cpm/μg) and 15,000 cpm of [^{32}P]-labeled cyclophilin cRNA (1×10^6 cpm/μg). Cyclophilin is expressed widely among tissues, including the brain, and its gene is most likely regulated in an "on or off" manner; cyclophilin mRNA was thus used as an internal standard for our measurements (Follesa et al., 1998). The hybridization reaction mixtures were incubated overnight at 50 °C and then subjected to digestion with RNase, after which RNA–RNA hybrids were detected by electrophoresis (on a sequencing gel containing 5% polyacrylamide and urea) and autoradiography. The amounts of $GABA_A$ receptor subunit mRNAs and cyclophilin mRNA were determined by measurement of the optical density of the corresponding bands on the autoradiogram with a densitometer (model GS-700; Bio-Rad, Hercules, CA), which was calibrated to detect saturated values so that all measurements were in the linear range. Data were normalized by dividing the optical density of the protected fragment for each receptor subunit mRNA by that of the respective protected fragment for cyclophilin mRNA. The amount of mRNA was therefore expressed in arbitrary units.

2.5. Immunocytofluorescence analysis

Hippocampal neurons cultured on coverslips were washed three times with phosphate-buffered saline, fixed for 1 h at room temperature with 4% paraformaldehyde in phosphate-buffered saline, washed three times with TN buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl], and permeabilized for 1 h at room temperature with TN-T buffer (0.1% Triton X-100 in TN buffer) containing 0.5% dried skim milk. Nonspecific binding sites for streptavidin and biotin were blocked by incubation of the cells for 15 min at room temperature with streptavidin blocking solution and then for an additional 15 min with biotin blocking solution (Vector, Burlingame, CA). The cells were then incubated overnight at 4 °C with goat polyclonal antibodies (1:500 dilution in TN-T buffer) to the $GABA_A$ receptor α1 (extracellular epitope, peptide N1–19) or α4 (extracellular epitope, peptide N1–19) subunit (Santa Cruz Biotechnology, Santa Cruz, CA). After several washes with TN-T buffer, the cells were incubated for 1 h at room temperature with biotin-conjugated donkey antibodies (1:200 in TN-T buffer) to goat immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) and then for 1 h with tetramethylrhodamine isothiocyanate-conjugated streptavidin (2 μg/ml in TN-T buffer; Jackson ImmunoResearch). The cells were washed extensively with TN buffer, and each coverslip was then positioned on a glass microscope slide with a permanent aqueous mounting medium (Sigma).

Epifluorescence imaging was obtained with an Olympus BX-41 microscope equipped with a UPlan FI 40× and 100× objective (numerical aperture, 1.30) and acquired with an F-View CCD camera.

Semiquantitative analysis for epifluorescence microscopy was performed with AnalySIS 3.2 software (Soft Imaging System, Münster, Germany); the acquired 8-bit gray-value images were white labeled on a black background, with a scale ranging from 0 as lower limit (black) to 255 as upper limit (white), so that the entire image histogram will be considered for calculating thresholds.

Each experiment was repeated three times, 5 fields were randomly selected in the coverslips of each experimental group. In each field, 20 cells were randomly selected by drawing a line surrounding the region of interest (ROI) in order to measure the intensity of fluorescence representing the abundance of the protein tested. The intensity mean ROI represents the integral image intensity which is the sum of all intensity of a ROI multiplied by the pixel area. Fluorescence intensity, which represents α1 or α4 subunit abundance, was eventually expressed in arbitrary units and compared as percentage change between the different experimental groups.

2.6. Whole-cell electrophysiological recording

Immediately before electrophysiological experiments, cover slips containing neurons were transferred to a perfusion chamber (Warner Instruments, Hampden, CT) and observed with a Nikon upright microscope equipped with Nomarski optics (40×). Large neurons with a pyramidal shape and well-defined dendritic processes were selected for recording. The membrane potential was clamped at −60 mV with an Axopatch 200-B amplifier (Axon Instruments, Foster City, CA). The resting membrane potential for the studied neurons was about −60 mV. Recording pipettes (borosilicate capillaries with a filament;

outer diameter, 1.5 mm) (Sutter Instruments, Novato, CA) were prepared with a two-step vertical puller (Sutter Instruments) and had resistances of 4–6 MΩ. Pipette capacitance and series resistance were compensated, the latter at 60%. Currents through the patch clamp amplifier were filtered at 2 kHz and digitized at 5.5 kHz with commercial software (pClamp 8.1; Axon Instruments).

The external solution contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES–NaOH (pH 7.3), and 11 mM glucose. The internal solution contained 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES–CsOH (pH 7.3), and 2 mM ATP (disodium salt). Drugs were applied with a fast-exchange flow-tube perfusion system driven by a motor (Warner Instruments). Agonists were applied at intervals of 30 s. All experiments were performed at room temperature (23–25 °C). Data were analyzed with pClampfit 8.01 software (Axon Instruments). Modulation of GABA-evoked Cl^- current by drugs is expressed as percentage potentiation, $[(I'/I)-1]\times 100\%$, where I is the average of GABA responses obtained before drug application and after drug washout, and I' is the average of the GABA-induced responses obtained from the same cell in the presence of drug.

2.7. Drug treatment of mice

Male CD-1 mice (Charles River, Como, Italy) with body masses of 20–30 g were maintained under an artificial 12-h light, 12-h dark cycle (light on 08:00–20:00 h) at a constant temperature of 23 ± 2 °C and 65% humidity. Food and water were freely available. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari.

Etizolam (1.5 mg/kg body mass, i.p.) and lorazepam (1.0 mg/kg, i.p.), each suspended in saline with one drop of Tween 80 per 5 ml, was administered to mice three times daily (08:00, 14:00, and 20:00 h) for 21 days. Control mice received an equivalent volume of vehicle. Thirty-six hours

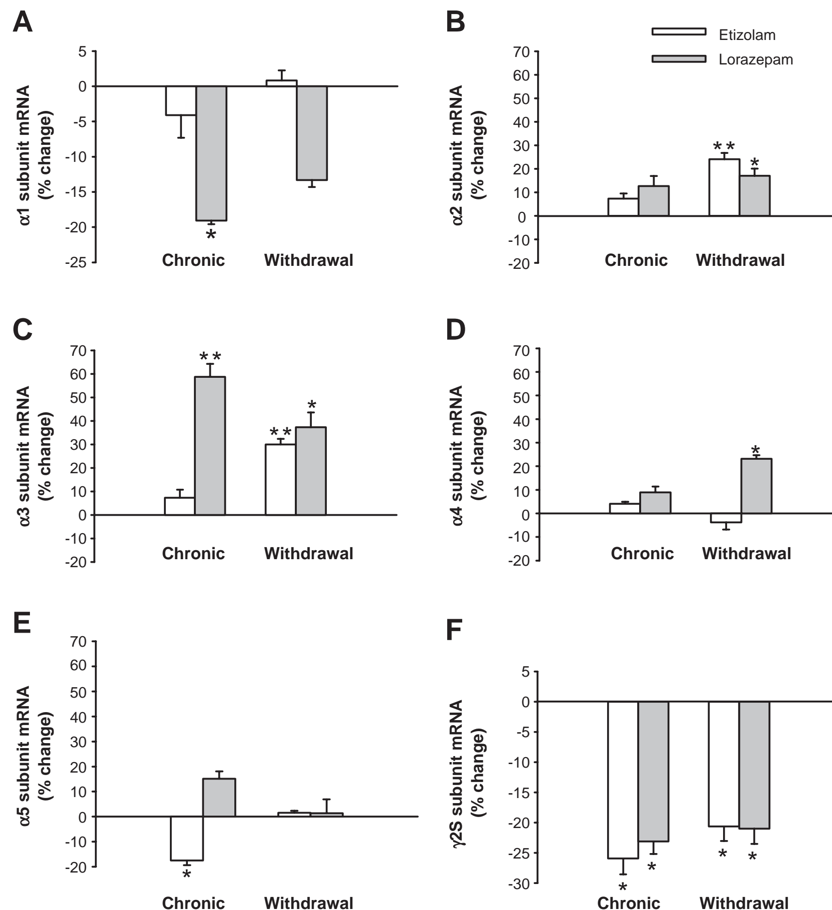

Fig. 1. Effects of chronic exposure to and subsequent withdrawal of etizolam or lorazepam on the abundance of $GABA_A$ receptor subunit mRNAs in cultured hippocampal cells. Cells were treated for 5 days with either 10 μM etizolam or 10 μM lorazepam and then incubated (or not) for 6 h in drug-free medium. The amounts of α1 (A), α2 (B), α3 (C), α4 (D), α5 (E), and γ2S (F) subunit mRNAs were determined by RNase protection assay. Data are means ± S.E.M. of 6–15 determinations in a total of three independent experiments and are expressed as percentage change relative to the corresponding value for control cultures incubated with vehicle for 5 days. *$P<0.05$, **$P<0.01$ versus control.

after the last injection of the chronic treatment protocol, mice were injected with either etizolam (3 mg/kg, i.p.), lorazepam (1 mg/kg, i.p.), or vehicle, each together with isoniazid (200 mg/kg, s.c.; Sigma) dissolved in distilled water. All mice were observed for 3 h for the onset of seizure activity and the pattern of seizures. A convulsant effect was defined as the induction of fully developed clonic seizures with loss of the righting reflex.

2.8. [^{35}S]TBPS binding

The cerebral cortex was dissected from mice subjected to chronic treatment and withdrawal of etizolam or lorazepam as described in Section 2.7 and the binding of *t*-butyl-[^{35}S]bicyclophosphorothionate ([^{35}S]TBPS) (DuPont Biotechnology System, Boston, MA) to cerebrocortical membranes was assayed as previously described (Squires et al., 1983), with the exception that nondialyzed membranes were used. The fresh brain tissue was homogenized with a Polytron PT 10 disrupter (setting 5 for 20 s) in 50 volumes of ice-cold 50 mM Tris-citrate buffer (pH 7.4 at 25 °C) containing 100 mM NaCl. The homogenate was centrifuged at 20,000 × *g* for 20 min at 4 °C, and the pellet was reconstituted in 50 volumes of Tris-citrate buffer for the binding assay. Specific binding of [^{35}S]TBPS (2 nM) to membranes derived from vehicle-treated mice was 50 ± 2 fmol/mg protein. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

2.9. Statistical analysis

Data are presented as means ± S.E.M. The statistical significance of differences was assessed by one-way analysis of variance followed by Scheffe's test, with the exception of behavioral data, which were analyzed by Fisher's exact probability test or Student's *t*-test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of chronic etizolam or lorazepam treatment on GABA$_A$ receptor gene expression

Cultured hippocampal neurons were exposed to 10 μM etizolam or 10 μM lorazepam for 5 days, after which the abundance of mRNAs for the α1, α2, α3, α4, and α5 subunits of the GABA$_A$ receptor as well as that of the mRNA for the short splice variant of the γ2 subunit (γ2S) were determined with an RNase protection assay. Chronic etizolam treatment did not significantly affect the abundance of the α1, α2, α3, or α4 subunit mRNAs (Fig. 1A–D). It did, however, induce significant decreases in the amounts of the α5 (−17.4 ± 1.9%) and γ2S (−25.9 ± 2.6%) subunit mRNAs relative to control values (Fig. 1E, F).

Long-term lorazepam treatment resulted in significant decreases in the amounts of the α1 (−19.1 ± 0.5%) and γ2S (−20.6 ± 2.4%) subunit mRNAs as well as a significant increase (58.8 ± 5.5%) in that of the α3 subunit mRNA (Fig. 1A, C, F). The same treatment did not affect the abundance of the α2, α4, and α5 subunit mRNAs (Fig. 1B, D, E).

3.2. Effects of etizolam or lorazepam withdrawal on GABA$_A$ receptor gene expression

To determine the effects of withdrawal of etizolam or lorazepam on GABA$_A$ receptor gene expression, we incubated hippocampal neurons first with 10 μM drug for 5 days and then in the absence of drug for 6 h. The abundance of the γ2S subunit mRNA, which was reduced after chronic etizolam treatment, remained significantly decreased, relative to control values, 6 h after removal of etizolam (Fig. 1F). In contrast, the amounts of the α2 and α3 subunit mRNAs were significantly increased (24.1 ± 2.6% and 30.0 ± 2.3%, respectively) by etizolam withdrawal (Fig. 1B, C). The abundance of the α5 subunit mRNA, which was decreased by chronic etizolam treatment, returned to control values after drug removal (Fig. 1E). Withdrawal of etizolam did not affect the abundance of the α1 or α4 subunit mRNAs (Fig. 1A, D).

Consistent with our previous observations (Follesa et al., 2001), the abundance of the γ2S subunit mRNA, which was reduced after chronic lorazepam treatment, remained significantly decreased, relative to control values, 6 h after drug removal (Fig. 1F). Similarly, the increase in the amount of the α3 subunit mRNA (Fig. 1C), but not the decrease in the amount of the α1 subunit mRNA (Fig. 1A), apparent after chronic lorazepam treatment persisted after drug withdrawal. Removal of lorazepam also induced significant increases in the abundance of the α2 and α4 subunit mRNAs (17.0 ± 3.1% and 23.1 ± 1.5%, respectively) (Fig. 1B, D), but it had no effect on that of the α5 subunit mRNA (Fig. 1E).

3.3. Effects of chronic exposure to and withdrawal of etizolam or lorazepam on α1 and α4 subunit abundance

In order to determine whether the changes in the abundance of α1 and α4 subunit mRNA induced by chronic exposure to and withdrawal of etizolam or lorazepam were associated with similar alterations in the amount of the encoded proteins, we subjected hippocampal neurons to immunocytofluorescence analysis with a fluorescence microscope.

As illustrated in Fig. 2, the average intensity of α1 subunit immunofluorescence staining was not significantly altered following chronic treatment with etizolam or 6 h after its discontinuation (Fig. 2B, C, F). On the contrary, α1 subunit immunofluorescence staining was reduced by 36 ± 4% and 26 ± 3% following chronic exposure to and withdrawal of lorazepam, respectively (Fig. 2D, E, F).

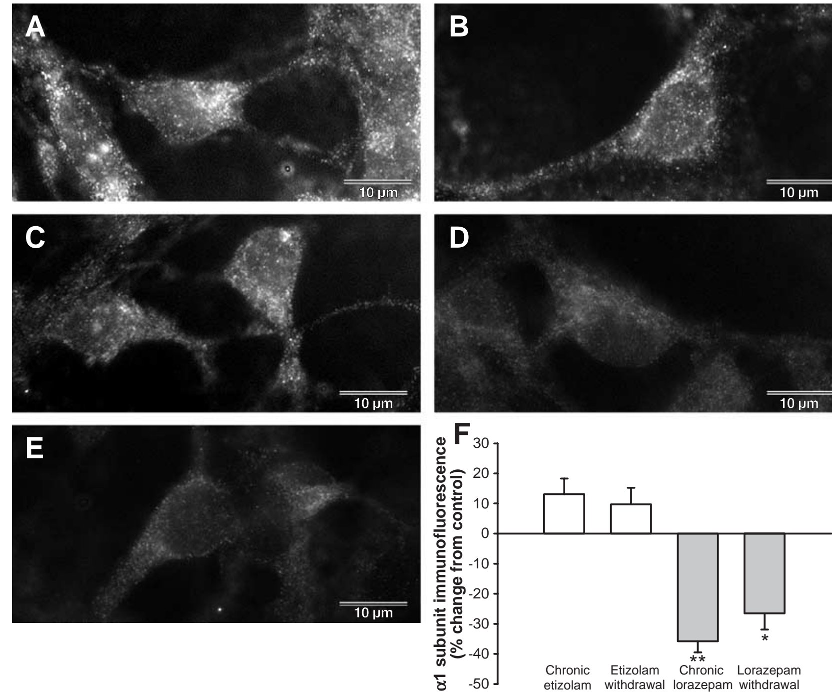

Fig. 2. Effects of chronic exposure to and subsequent withdrawal of etizolam or lorazepam on α1 subunit immunofluorescence in cultured hippocampal cells. Cells were treated for 5 days with vehicle (A) or either 10 μM etizolam (B) or 10 μM lorazepam (D) and then incubated for 6 h in etizolam- (C) or lorazepam- (E) free medium. Images were obtained using a 100× objective (scale bar = 10 μm). (F) Summary graph showing the results of quantification of α1 subunit immunofluorescence intensity in cultures belonging to the different treatment groups. Data were normalized with respect to control and are expressed as percentage change from control value ± S.E.M. of 300 neurons from 15 coverslips per treatment group. *$P<0.05$, **$P<0.01$ versus control.

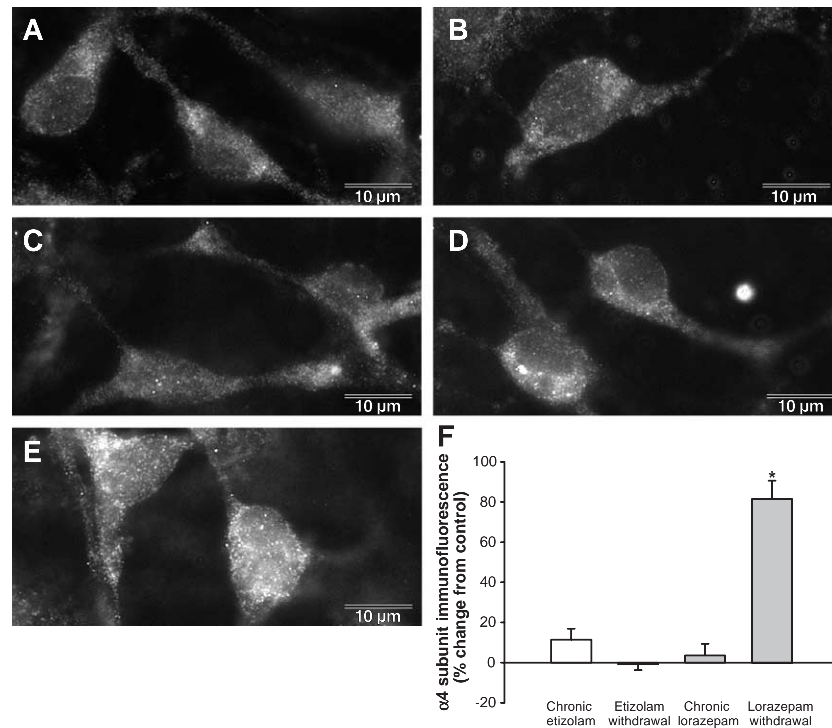

Fig. 3. Effects of chronic exposure to and subsequent withdrawal of etizolam or lorazepam on α4 subunit immunofluorescence in cultured hippocampal cells. Cells were treated for 5 days with vehicle (A) or either 10 μM etizolam (B) or 10 μM lorazepam (D) and then incubated for 6 h in etizolam- (C) or lorazepam- (E) free medium. Images were obtained using a 100× objective (scale bar = 10 μm). (F) Summary graph showing the results of quantification of α4 subunit immunofluorescence intensity in cultures belonging to the different treatment groups. Data were normalized with respect to control and are expressed as percentage change from control value ± S.E.M. of 300 neurons from 15 coverslips per treatment group. *$P<0.001$ versus control.

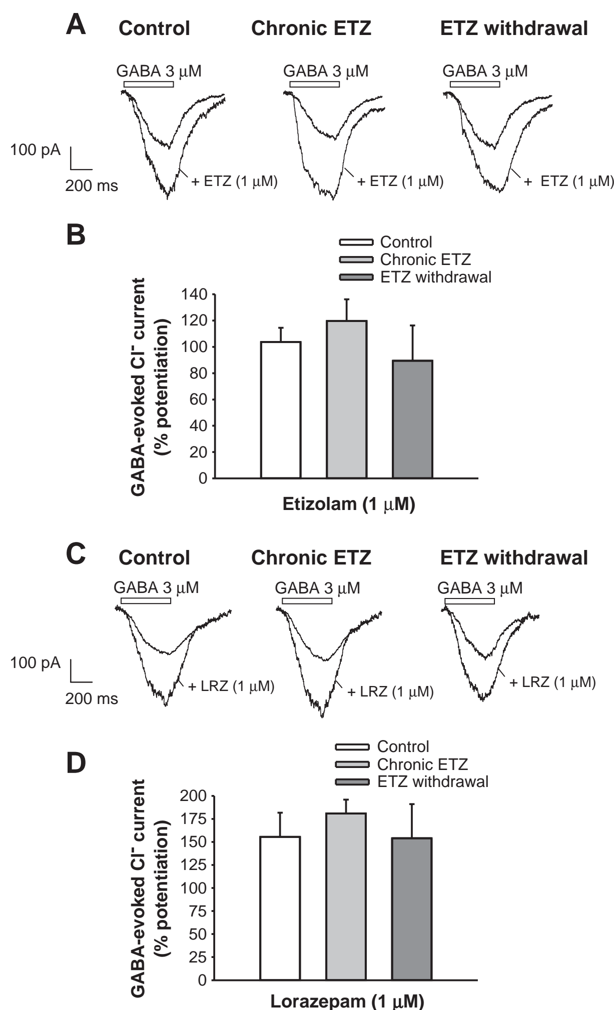

Fig. 4. Acute modulatory effects of etizolam or lorazepam on $GABA_A$ receptor function in hippocampal neurons subjected to chronic exposure to or withdrawal of etizolam. The acute effects of 1 μM etizolam (A, B) or 1 μM lorazepam (C, D) on the Cl^- current evoked by GABA (3 μM) were determined in control neurons and in those subjected to chronic treatment with or withdrawal of etizolam (10 μM). Representative current traces are shown in (A) and (C). Data in (B) and (D) are expressed as percentage potentiation of the GABA response and are means ± S.E.M. of values from 10 to 16 neurons in 4 independent experiments. ETZ, etizolam; LRZ, lorazepam.

The average intensity of α4 subunit immunofluorescence staining did not change after etizolam chronic treatment or withdrawal for 6 h (Fig. 3B, C, F). The same parameter was also not altered following chronic exposure to lorazepam, but resulted significantly enhanced 6 h after lorazepam withdrawal (Fig. 3D, E, F).

3.4. *Effects of chronic exposure to and withdrawal of etizolam on acute etizolam, lorazepam, or flumazenil modulation of $GABA_A$ receptor function*

We next examined the impact of the changes in subunit mRNA abundance induced by chronic exposure to and withdrawal of etizolam on the sensitivity of $GABA_A$ receptors to the acute modulatory effects of etizolam, lorazepam, and flumazenil in hippocampal neurons. In control neurons, etizolam (1 μM) or lorazepam (1 μM) potentiated the Cl^- current induced by 3 μM GABA by 104 ± 11% and 155 ± 26%, respectively (Fig. 4). The efficacy of these two drugs was not significantly altered either by chronic etizolam treatment or by subsequent etizolam withdrawal. Consistent with its pharmacological profile as a pure benzodiazepine receptor antagonist devoid of intrinsic modulatory activity, flumazenil (3 μM) did not significantly affect GABA-evoked Cl^- current in control neurons (Fig. 5). Furthermore, this lack of activity of flumazenil was not affected by either chronic etizolam treatment or etizolam withdrawal.

3.5. *Effects of chronic exposure to and withdrawal of lorazepam on acute lorazepam or flumazenil modulation of $GABA_A$ receptor function*

Consistent with our previous results (Follesa et al., 2001), chronic exposure of hippocampal neurons to lorazepam resulted in a significant reduction (by 48%) in the efficacy of a challenge concentration of lorazepam (1 μM) with regard to potentiation of GABA-evoked Cl^- current (Fig. 6). In neurons subjected to lorazepam withdrawal, however, the modulatory efficacy of lorazepam was restored to a level similar to that apparent in control neurons.

Whereas flumazenil (3 μM) did not affect GABA-evoked Cl^- current in either control neurons or cells subjected to

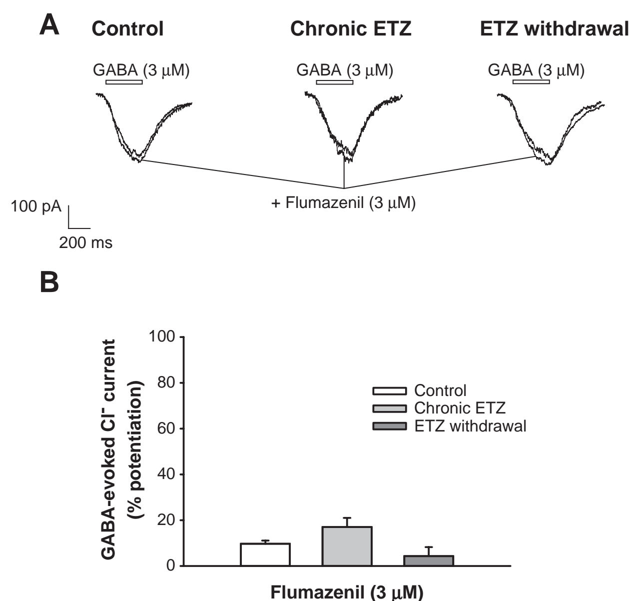

Fig. 5. Acute modulatory effect of flumazenil on $GABA_A$ receptor function in hippocampal neurons subjected to chronic exposure to or withdrawal of etizolam. (A) Representative tracings of Cl^- currents induced by GABA (3 μM) in the absence or presence of flumazenil (3 μM) in a control cell, a cell exposed to 10 μM etizolam for 5 days, and a cell subjected to etizolam withdrawal for 6 h. (B) Quantitation of the modulatory effect of flumazenil on GABA-evoked Cl^- current in cells of the three experimental groups. Data are expressed as percentage potentiation of the GABA response and are means ± S.E.M. of values from 6 to 10 neurons in 3 independent experiments.

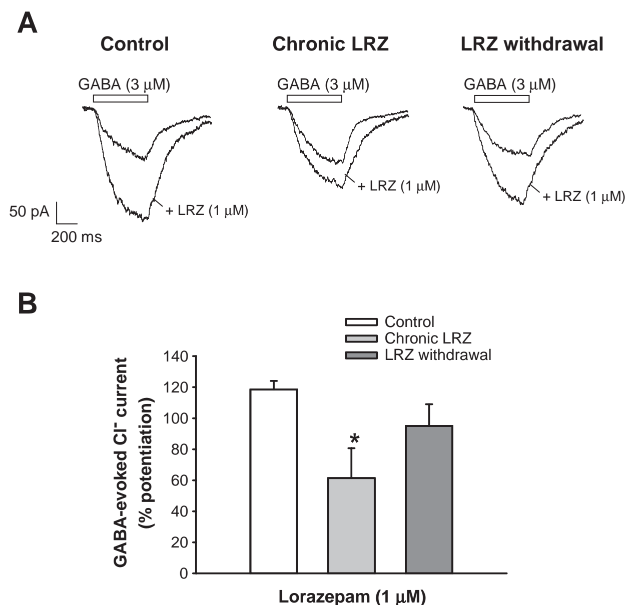

Fig. 6. Acute modulatory effect of lorazepam on $GABA_A$ receptor function in hippocampal neurons subjected to chronic exposure to or withdrawal of lorazepam. (A) Representative tracings of Cl^- currents induced by GABA (3 μM) in the absence or presence of lorazepam (1 μM) in a control cell, a cell exposed to 10 μM lorazepam for 5 days, and a cell subjected to lorazepam withdrawal for 6 h. (B) Quantitation of the modulatory effect of lorazepam on GABA-evoked Cl^- current in the three experimental groups. Data are expressed as percentage potentiation of the GABA response and are means ± S.E.M. of values from 8 to 12 neurons in 4 independent experiments. *$P<0.05$ versus value for control cells.

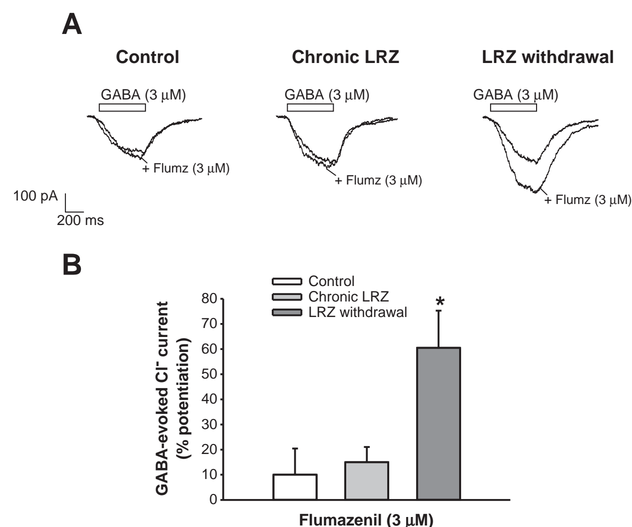

Fig. 7. Acute modulatory effect of flumazenil on $GABA_A$ receptor function in hippocampal neurons subjected to chronic exposure to or withdrawal of lorazepam. (A) Representative tracings of Cl^- currents induced by GABA (3 μM) in the absence or presence of flumazenil (3 μM) in a control cell, a cell exposed to 10 μM lorazepam for 5 days, and a cell subjected to lorazepam withdrawal for 6 h. (B) Quantitation of the modulatory effect of flumazenil on GABA-evoked Cl^- current in the three experimental groups. Data are expressed as percentage potentiation of the GABA response and are means ± S.E.M. of values from 6 to 10 neurons in 3 independent experiments. *$P<0.01$ versus value for control cells.

long-term treatment with lorazepam, it markedly potentiated (by 60 ± 15%) the GABA response in neurons subjected to lorazepam withdrawal (Fig. 7).

3.6. Effects of chronic treatment with etizolam or lorazepam on their ability to antagonize convulsions induced by isoniazid

Etizolam (3 mg/kg, i.p.) or lorazepam (1 mg/kg, i.p) antagonized convulsions induced by isoniazid (200 mg/kg, s.c.) in mice chronically treated with vehicle (Table 1). The protective effects of the two drugs were similar, with each reducing the number of animals manifesting convulsions by ~90%. Moreover, etizolam and lorazepam each significantly delayed the onset of seizures (85 ± 6 and 90 ± 5 min, respectively, compared with 47 ± 5 min in control animals). Long-term treatment with etizolam (1.5 mg/kg, i.p., three times a day for 21 days) reduced only slightly the anticonvulsant action of this drug. A challenge dose (3 mg/kg, i.p.) of etizolam administered 36 h after the last injection of the chronic treatment protocol thus still significantly antagonized the induction of seizure activity and death by isoniazid. Consistent with previous observations (Miller et al., 1988), chronic administration of lorazepam (1 mg/kg, i.p., three times daily for 21 days) resulted in an ~50% decrease in the anticonvulsant activity of this drug, although a challenge dose (1 mg/kg, i.p.) was still able to significantly increase the latency of convulsions and to reduce the number of animals that died. The latency of isoniazid-induced convulsions was significantly decreased in the animals chronically treated with lorazepam

Table 1
Effects of chronic treatment with etizolam or lorazepam on the anticonvulsant action of the respective drugs in mice exposed to isoniazid

Challenge drug	Latency of convulsions (min)	No. of animals with convulsions	No. of animals dying
Chronic vehicle			
ISO	47 ± 5	16/16 (100%)	15/16 (94%)
ISO + ETZ	85 ± 6[a]	2/17[b] (12%)	1/17[b] (6%)
ISO + LRZ	90 ± 5[a]	2/18[b] (11%)	0/18[b] (0%)
Chronic ETZ			
ISO	62 ± 4[c]	18/18 (100%)	13/18 (72%)
ISO + ETZ	79 ± 2[a]	7/18[b] (39%)	2/18[b] (11%)
Chronic LRZ			
ISO	38 ± 4[c]	18/18 (100%)	18/18 (100%)
ISO + LRZ	117 ± 21[a]	10/18[b] (56%)	4/18[b] (22%)

Isoniazid (ISO, 200 mg/kg, s.c.) and either etizolam (ETZ, 3 mg/kg, i.p.) or lorazepam (LRZ, 1 mg/kg, i.p.) were administered 36 h after the last injection of the chronic treatment protocol with vehicle, etizolam, or lorazepam. Animals were observed for 3 h for the onset of seizure activity, the pattern of seizures, and death. Data are means ± S.E.M. for the indicated numbers of animals.

[a] $P<0.01$ versus respective group treated with isoniazid (Student's t test).
[b] $P<0.005$ versus respective group treated with isoniazid (Fisher's exact probability test).
[c] $P<0.05$ versus chronic vehicle+isoniazid (Student's t test).

Table 2
[^{35}S]TBPS binding to cerebrocortical membranes prepared from mice at various times after discontinuation of long-term treatment with lorazepam or etizolam

Treatment	[^{35}S]TBPS binding (% of control)		
	12 h	48 h	96 h
Lorazepam	118±2[a]	127±3[a]	74±2[a]
Etizolam	110±3	110±2	109±1

Data are expressed as a percentage of the value for control mice treated with vehicle and are means±S.E.M. for 10 animals per group.
[a] $P<0.05$ versus control.

(38 versus 47 min) but was increased in those subjected to long-term treatment with etizolam (62 versus 47 min).

3.7. Effects of etizolam or lorazepam withdrawal on [^{35}S]TBPS binding

Finally, we examined the effects of withdrawal of etizolam or lorazepam on the binding of [^{35}S]TBPS to cerebrocortical membranes prepared from mice 12, 48, or 96 h after discontinuation of long-term treatment. [^{35}S]TBPS binding was significantly increased 12 and 48 h after discontinuation of lorazepam treatment but it was significantly decreased at 96 h (Table 2). In contrast, withdrawal of etizolam after long-term treatment had no effect on [^{35}S]TBPS binding at any of the time points examined.

4. Discussion

One of the major problems limiting the long-term use of benzodiazepines for the clinical treatment of anxiety disorders, insomnia, and epilepsy is the development of tolerance and physical dependence. Tolerance is associated with a reduced sensitivity of $GABA_A$ receptors to the positive modulatory action of benzodiazepines (Bateson, 2002; File, 1985; Ryan and Boisse, 1983), and this reduced sensitivity is thought to result from changes in the transcription of genes for specific receptor subunits (Follesa et al., 2001; Impagnatiello et al., 1996; Kang and Miller, 1991; Li et al., 2000; Tietz et al., 1999). Such changes in gene transcription would be expected to affect the subunit composition of $GABA_A$ receptors present in the neuronal membrane and thereby to alter the functional and pharmacological properties of the receptors. Our previous studies of long-term treatment with benzodiazepine receptor ligands endowed with high intrinsic efficacy (such as diazepam) or low intrinsic efficacy (such as imidazenil) have suggested that the level of intrinsic efficacy is an important determinant of the chronic effects of these drugs on $GABA_A$ receptor gene expression (Follesa et al., 2001).

We have now examined the long-term effects of etizolam, in comparison with those of the classical benzodiazepine lorazepam, on $GABA_A$ receptor gene expression and function. Etizolam possesses a pharmacological profile similar to that of classical benzodiazepines (Nakanishi et al., 1972; Tsumagari et al., 1978), but it differs from these ligands in that its intrinsic efficacy and potency at $GABA_A$ receptors containing the α1 subunit are markedly reduced (Sanna et al., 1999). In addition, the administration of etizolam to patients diagnosed with generalized anxiety disorder is associated with a lower liability for the induction of tolerance and dependence as well as a reduced sedative effect when compared with treatment with classical benzodiazepines (Casacchia et al., 1990; Savoldi et al., 1990).

Our results now show that chronic treatment with and subsequent withdrawal of etizolam induce changes in $GABA_A$ receptor gene expression in cultured hippocampal neurons that differ markedly from those elicited by lorazepam. Furthermore, the molecular changes induced by chronic exposure to etizolam were not accompanied by significant alterations in the pharmacological sensitivity of $GABA_A$ receptors to the acute action of this drug, suggesting that such treatment may not be associated with pharmacodynamic tolerance. Long-term treatment of hippocampal cells with lorazepam induced significant decreases in the amounts of the α1 and γ2S subunit mRNAs as well as a decrease in receptor sensitivity to acute positive modulation by this drug. This functional uncoupling between the GABA-activated ionophore and the allosteric benzodiazepine recognition site of the $GABA_A$ receptor might thus be attributable to down-regulation of the α1 and γ2S subunits. Receptors containing these two subunits constitute the predominant receptor subtype (~45% of all $GABA_A$ receptors) in the mammalian central nervous system (Barnard et al., 1998; McKernan and Whiting, 1996) and are important for optimal pharmacological sensitivity to benzodiazepine receptor agonists (Barnard et al., 1998).

Long-term treatment with etizolam also induced a significant decrease in the abundance of the α5 and γ2S subunit mRNAs but did not affect that of the α1 subunit mRNA. Given that such chronic treatment with etizolam was not associated with significant changes in the modulatory efficacy of etizolam or lorazepam, these results suggest that down-regulation of the α5 and γ2S subunits is not sufficient to produce pharmacodynamic tolerance to these drugs.

Discontinuation of long-term exposure of hippocampal neurons to lorazepam resulted in restoration of the acute positive modulatory effect of this drug at $GABA_A$ receptors, despite the fact that the abundance of the γ2S subunit mRNA remained significantly decreased; the amount of the α1 subunit mRNA also remained decreased but the difference with control cells was no longer significant. Given that classical benzodiazepines such as lorazepam are not selective with regard to $GABA_A$ receptors containing α1, α2, α3, or α5 subunits, possessing high affinity for all of these receptor subtypes (Sieghart, 1995), it is possible that the increase in the abundance of the α2 and α3 subunit mRNAs associated with lorazepam withdrawal serves to compensate for the loss of α1 subunit-containing receptors.

Increased abundance of the α2 and α3 subunit mRNAs was also observed in association with withdrawal of etizolam. However, these changes did not appear to contribute to affect the sensitivity to etizolam or lorazepam, as the acute modulatory action of these drugs was not altered, and their relevance still remains to be determined.

Withdrawal of lorazepam also induced a marked increase in the level of the α4 subunit mRNA. To determine whether this effect was accompanied by an increased surface expression of $GABA_A$ receptors containing this subunit, we tested the action of flumazenil, a competitive benzodiazepine receptor antagonist devoid of intrinsic activity at receptors that contain α1, α2, α3, or α5 subunits but endowed with agonist-like activity at those containing the α4 subunit (Wafford et al., 1996; Whittemore et al., 1996). Whereas flumazenil was ineffective in modulating GABA-evoked Cl^- current in control cells or in cells chronically exposed to lorazepam, it potentiated the GABA response in cells subjected to lorazepam withdrawal, suggesting that newly assembled α4 subunit-containing receptors are indeed expressed in the membrane of these latter cells.

Up-regulation of α4 subunit gene transcription has also been demonstrated in response to withdrawal of neurosteroids (Follesa et al., 2000, 2003; Smith et al., 1998) or of ethanol (Cagetti et al., 2003; Devaud et al., 1995; Sanna et al., 2003) as well as in response to electrical kindling in the hippocampus of rats, a procedure that also reduces both inhibitory GABA-mediated transmission and seizure threshold (Kamphuis et al., 1995). Increased expression of $GABA_A$ receptors containing the α4 subunit might thus be important in the development of cellular hyperexcitability, which, in humans and animals, is associated with anxiety-like behavior.

In contrast to the effects of lorazepam withdrawal, the abrupt discontinuation of long-term exposure to etizolam neither induced an increase in the amount of the α4 subunit mRNA nor conferred on flumazenil the ability to enhance the electrophysiological response to GABA. These results thus further suggest that the differences in the changes in $GABA_A$ receptor gene expression and receptor function induced by the chronic administration of etizolam or lorazepam may contribute to a higher threshold of excitability and explain the reduced severity of symptoms of withdrawal syndrome associated with the former drug.

Our results with mice chronically treated with etizolam or lorazepam are consistent with the molecular and functional data. Long-term treatment with etizolam, unlike that with lorazepam, did not induce substantial tolerance or down-regulation of inhibitory GABA-mediated transmission. In mice chronically treated with etizolam, a challenge dose of this drug administered 36 h after the last treatment antagonized isoniazid-induced seizure activity with an efficacy similar to that observed in animals chronically treated with vehicle. Liability to produce tolerance and dependence is thought to be related directly to the intrinsic efficacy of an agonist (Costa, 1998; Woods et al., 1992). Prolonged activation of $GABA_A$ receptor function induced by benzodiazepine receptor agonists with a high intrinsic efficacy thus results in marked tolerance, whereas partial agonists such as imidazenil induce a much reduced level of tolerance or none at all (Auta et al., 1994; Ghiani et al., 1994). We have previously shown that both the potency and efficacy of etizolam in potentiation of GABA-evoked Cl^- current at α1β2γ2S $GABA_A$ receptors are lower than those of classical benzodiazepines (Sanna et al., 1999). Given that the α1 subunit is implicated both in the sedative and anticonvulsant effects of diazepam (Rudolph et al., 1999) as well as in the development of tolerance to flurazepam (O'Donovan et al., 1992; Tietz et al., 1999), the reduced intrinsic efficacy of etizolam at α1-containing receptors might contribute to the lack of development of a marked degree of tolerance to the anticonvulsant effect of this drug and to its low-level sedative action in patients.

Finally, the failure of etizolam to induce tolerance to its anticonvulsant effect in mice is consistent with our observation that withdrawal of etizolam after its chronic administration did not induce significant changes in $[^{35}S]$TBPS binding to cerebrocortical membranes. In contrast, $[^{35}S]$TBPS binding was significantly increased 12 and 48 h after lorazepam withdrawal, consistent with an enhanced expression of $GABA_A$ receptors containing the α4 subunit, which have been suggested to represent a $GABA_A$ receptor subtype that may contribute to a heterogeneous population of atypical GABA-insensitive $[^{35}S]$TBPS binding sites (Sinkkonen et al., 2001a,b). On the contrary, the failure of withdrawal from chronic etizolam treatment to increase $[^{35}S]$TBPS binding thus suggests that such withdrawal does not induce similar changes in $GABA_A$ receptor subtypes.

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