Glutamic acid decarboxylase and glutamate receptor changes during tolerance and dependence to benzodiazepines

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Protracted administration of diazepam elicits tolerance, whereas discontinuation of treatment results in signs of dependence. Tolerance to the anticonvulsant action of diazepam is present in an early phase (6, 24, and 36 h) but disappears in a late phase (72–96 h) of withdrawal. In contrast, signs of dependence such as decrease in open-arm entries on an elevated plus-maze and increased susceptibility to pentylenetetrazol-induced seizures were apparent 96 h (but not 12, 24, or 48 h) after diazepam withdrawal. During the first 72 h of withdrawal, tolerance is associated with changes in the expression of $GABA_A$ (γ-aminobutyric acid type A) receptor subunits (decrease in γ_2 and α_1; increase in α_5) and with an increase of mRNA expression of the most abundant form of glutamic acid decarboxylase (GAD), GAD_{67}. In contrast, DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor GluR1 subunit mRNA and cognate protein, which are normal during the early phase of diazepam withdrawal, increase by approximately 30% in cortex and hippocampus in association with the appearance of signs of dependence 96 h after diazepam withdrawal. Immunohistochemical studies of GluR1 subunit expression with gold-immunolabeling technique reveal that the increase of GluR1 subunit protein is localized to layer V pyramidal neurons and their apical dendrites in the cortex, and to pyramidal neurons and in their dendritic fields in hippocampus. The results suggest an involvement of GABA-mediated processes in the development and maintenance of tolerance to diazepam, whereas excitatory amino acid-related processes (presumably via AMPA receptors) may be involved in the expression of signs of dependence after withdrawal.

Diazepam, alprazolam, triazolam, lorazapam, or flurazepam, all ligands of benzodiazepine recognition sites (BZ-RS) expressed by the γ-aminobutyric acid type A ($GABA_A$) receptors, are used in psychiatry and neurology to treat chronic disorders such as epilepsy, sleep disturbance, and generalized anxiety, often requiring a long-term administration. The protracted administration of BZ-RS ligands acting as full positive allosteric modulators (FAM) of GABA action at $GABA_A$ receptors results in a number of undesirable effects including tolerance and physical dependence (1–3). Tolerance to these drugs occurs after a well defined period of treatment, and the onset varies according to the type of BZ-RS ligand and the disease being treated. For example, during a protracted treatment with BZ-RS ligands acting as FAMs, sedation is the first response to show tolerance, followed by amnesia, anticonvulsant action, and ultimately anxiolytic activity (1–3). This different time course, *inter alia*, might reflect differences in the transcription time and assembly of one or more of the 17 genes that encode the various $GABA_A$ receptor subunits (4–9). The abrupt appearance of signs of dependence a few days after discontinuation of BZ-RS ligands protracted treatment (1, 2, 10, 11) is another unwanted side effect of protracted treatment with high doses of these drugs.

Whereas tolerance to the actions of BZ-RS ligands has been associated with changes in $GABA_A$ receptor subunit assembly (12–18) or allosteric subunit uncoupling (19, 20), the molecular mechanisms underlying dependence after withdrawal of these drugs have not been well characterized. Dependence does not likely involve down-regulation of $GABA_A$ receptors because the modification of $GABA_A$ receptor subunit assembly or coupling and the down-regulation of GABAergic function associated with tolerance disappear before the onset of the withdrawal syndrome (14, 21, 22). In 1993, Turski and colleagues (11), and successively others (23, 24), suggested that an enhanced glutamatergic transmission is a possible component of BZ-RS ligands withdrawal syndrome characterized by tremors, myoclonic jerks, wet dog shakes, piloerection, loss of body weight, excitability, anxiety, and electroencephalographic seizure pattern. Pharmacological experiments with agonists or antagonists of specific glutamate receptor subtypes suggested that whereas *N*-methyl-D-aspartate (NMDA)-dependent mechanisms may underlie the expression of signs of dependence, AMPA receptor activation may be essential for the induction of the withdrawal syndrome following a protracted diazepam administration. Taken together, these data suggest that: (*i*) the tolerance to the actions of BZ-RS ligands acting as FAMs may be associated with changes in GABAergic transmission; (*ii*) the dependence elicited by these drugs may be associated with an enhanced glutamatergic transmission; and (*iii*) an enhanced glutamatergic transmission may be a common mechanism underlying the abrupt discontinuation of protracted treatment with GABAergic transmission enhancers.

We tested this hypothesis by studying the time course for the duration of tolerance and for the emergence of withdrawal signs following discontinuation of a 14-day repeated diazepam treatment by using the elevated plus-maze test (25) and the susceptibility to bicuculline (26) or pentylenetetrazol (PTZ)-induced seizures. To elucidate the molecular mechanisms underlying tolerance and dependence, we concurrently quantified in frontal and occipital cortices and hippocampi of rats the expression of mRNA encoding for two isoforms of glutamic acid decarboxylase [glutamic acid decarboxylase 67 (GAD_{67}) or GAD_{65}], for AMPA (GluR1, GluR2, GluR3, and GluR4) receptor subunits, and for the NR1 NMDA receptor subunit (studied with primers recognizing all NR1 transcripts) by using a quantitative reverse transcription–PCR (RT-PCR) technique with appropriate mutated internal standards (27).

Materials and Methods

Animals, Drugs, and Reagents. Male Fisher 344 rats (Harlan, Indianapolis) weighing 120–130 g were maintained on a 12-hr light/dark cycle with free access to food and water. All exper-

Abbreviations: AMPA, DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; BZ-RS, benzodiazepine recognition site; FAM, full positive allosteric modulator; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazol; RT-PCR, reverse transcription–PCR;

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iments were carried out in accordance with the National Institute of Health, *Guide for the Care and Use of Laboratory Animals*.

Diazepam was from Hoffmann-La Roche; PTZ from Sigma. The Hot Tub DNA polymerase, Human placenta Ribonuclease Inhibitor, *Bgl*II, *Xba*I, and [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) were from Amersham Pharmacia. Random hexamers were from Amersham Pharmacia. Moloney murine leukemia virus (MMLV) reverse transcriptase, CsCl, guanidine isothiocyanate, agarose, and low-melting-point agarose were from GIBCO/BRL.

Schedule for Long-Term Diazepam Treatment. Diazepam was suspended in water containing 0.05% Tween-20 and administered in 1 ml volume by oral gavage three times daily (at approximately 9:00 a.m., 2:00 p.m., and 7:00 p.m.) for 14 days at increasing doses (days 1–3, 17.6 μmol/kg; days 4–6, 35.2 μmol/kg; days 7–10, 52.8 μmol/kg; and days 11–14, 70.4 μmol/kg). Control rats received only vehicle.

Anticonvulsant Tolerance Test. Rats receiving long-term diazepam or vehicle treatment were withdrawn for 6, 24, 36, and 72 h before receiving a standard oral challenge with diazepam (17.6 μmol/kg). The administration of diazepam was followed 30 min later by the bicuculline i.v. infusion (convulsion test) (26).

Dependence Liability Test. The emergence of a withdrawal syndrome was determined by using the elevated plus-maze test and the susceptibility to PTZ-induced seizure at 12 and 96 h after termination of a 14-day diazepam or vehicle administration. The apparatus used for the elevated plus-maze test was as described by Auta *et al.* (25). The rat exploratory activities (number of entries and time spent) on the open and closed arms of the maze were recorded over a period of 5 min. For the PTZ-induced clonic seizure test, rats received i.v. infusion of PTZ (50 μmol/ml) at a rate of 0.46 ml/min (26). The infusion was stopped at the first visual sign of tonic-clonic seizures. The time, in seconds, between the start of the infusion and induction of tonic-clonic seizures was used to calculate the PTZ threshold dose (μmol/kg) necessary to induce tonic-clonic seizures.

RNA Isolation and Quantitative RT-PCR Analysis. The hippocampi, frontal (from 6.7 to 3.2 mm anterior to Bregma), and occipital (5.3 to 8 mm posterior to Bregma) cortices, as well as cerebellum, were removed and frozen on dry ice. Total RNA was isolated as described in ref. 12. Quantitative RT-PCR analysis was performed by using mutated internal standards as described in ref. 27. Primers for GAD_{67} mRNA were: forward, 1855–1878 bp; reverse, 2246–2269 bp (GenBank accession no. M81883); internal standard contained a *Bgl*II restriction endonuclease that on digestion generated fragments of 199- and 216-bp. Primers for GAD_{65} mRNA were: forward, 82–103; reverse 507–532 (GenBank accession no. M72422); internal standard contained an *Xba*I restriction endonuclease site that on digestion generated fragments of 215- and 235-bp. The primer pairs for the determination of GluR1, GluR2, GluR3, and GluR4 and their respective internal standards were designed to allow amplification of 300–380 bp and to include cDNA sequences that comprise the Flip/Flop segment for all four subunits as described in detail by Longone *et al.* (28). Primers for the NR1 subunit mRNAs were: forward, 3280–3312; reverse, 3677–3698 (GenBank accession no. X63255); internal standard contained a *Bgl*II restriction endonuclease site that on digestion generated fragments of 220- and 198-bp. These primers include the cDNA sequence that comprises all eight NR1 splice variants.

Western Blot Analyses of GluR1 Subunit. Brain tissue homogenates, after SDS/10% PAGE, were blotted onto nitrocellulose membrane and probed with antibodies specific for the GluR1 subunit (28). The blot was reprobed with a β-actin antibody (28).

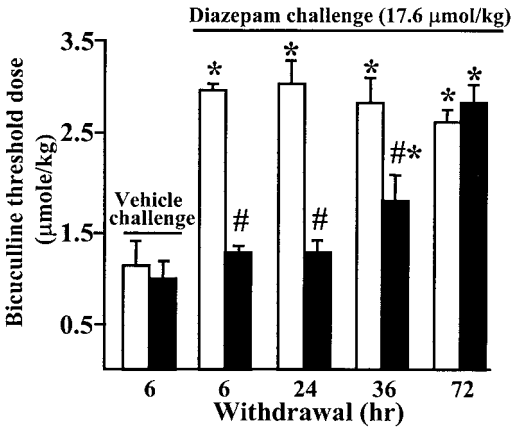

Fig. 1. Evolution of diazepam anticonvulsant tolerance in diazepam-withdrawn rats. Rats receiving 14 days treatment with vehicle (□) or diazepam (■) were withdrawn for different times before receiving orally a vehicle or a diazepam challenge, 30 min before bicuculline-induced seizure test (25). Values are mean ± SE of 5–6 rats. *, $P < 0.0.01$ when compared with vehicle challenge groups; #, $P < 0.01$ when long-term diazepam-treated (■) rats were compared with the corresponding long-term vehicle-treated (□) rats. (ANOVA followed by Duncan's multiple comparison test)

Gold-Immunolabeling of GluR1 Receptor Subunit Protein. The expression of the polypeptide for GluR1 AMPA receptor subunit was measured immunohistochemically, with antibodies linked to 1-nm gold particles. Gold-immunohistochemical studies were conducted in rats perfused with fixatives as described in detail by Pesold *et al.* (29). Briefly, 20-μm sections were incubated for 18 h with the primary antibody. Following several rinses, sections were incubated with a secondary antibody tagged with 1-nm colloidal gold particles for 1 h (diluted 1:200 in 1% BSA). Sections received a 12-min silver enhancement of the gold particles for visualization at light microscopy. Sections were then counterstained with toluidine blue, dried, and coverslipped. Control sections were included in which the primary antibody was omitted. By using a Zeiss Axioskope with a 100X oil-immersion objective, the number of gold particles per 100-μm^2 were counted by the grain counting program of the Samba 2000 system (Imaging Products International, Chantilly, VA). "Specific-labeling" was calculated as the total number of gold particles minus the number of gold particles in the control sections ("nonspecific" labeling), in which the same protocol was followed except that the primary antibody was omitted.

Results

Tolerance and Dependence After Long-Term Treatment with Diazepam. *Tolerance.* To establish the time course of the disappearance of anticonvulsant tolerance in diazepam-withdrawn rats (6, 12, 24, 36, and 72 h), we determined the anticonvulsant action of a challenging standard dose (17.6 μmol/kg *per os*) of diazepam by measuring the threshold dose of bicuculline necessary to elicit tonic-clonic convulsions. In vehicle-withdrawn rats this standard dose of diazepam increased the convulsive threshold dose of bicuculline by approximately 3-fold (Fig. 1). When administered 6 and 24 h after diazepam withdrawal, this dose of diazepam failed to modify the threshold dose of bicuculline that elicited convulsions suggesting that diazepam tolerance persists. In contrast, tolerance to diazepam decreased at 36 h and completely disappeared 72 h after the termination of long-term treatment with diazepam (Fig. 1).

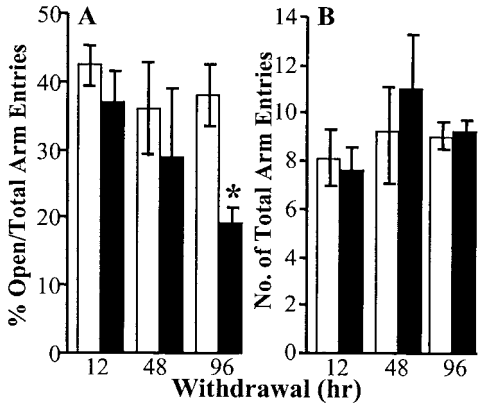

Fig. 2. Elevated-plus maze test following long-term vehicle-treatment (□) or diazepam-treatment (■) withdrawal. Values are mean ± SEM of 5–6 rats. *, $P < 0.01$ when compared with the respective vehicle-treated rats. Nonparametric analysis by using the Kruskal-Wallis test followed by the Mann–Whitney *U* test.

Dependence. To determine the time-course for the appearance of diazepam withdrawal-induced anxiety, rats were subjected to an elevated plus-maze test. Figure 2*A* shows a significant decrease in the percentage of open-arm entries 96 h after diazepam-withdrawal. The total maze activity (Fig. 2*B*) of these diazepam-withdrawn rats was not decreased. To define whether alteration in $GABA_A$ receptor sensitivity occurs during diazepam withdrawal, we measured susceptibility to PTZ-induced seizures in rats withdrawn from vehicle or diazepam treatment 12 or 96 h before the test. The threshold dose for PTZ-induced seizures was significantly lower (293 ± 8.4 μmol/kg, $P < 0.05$; $n = 5$) 96 h, but not 12 h (320 ± 7.8 μmol/kg), after diazepam withdrawal when compared with the vehicle-withdrawn group of rats (350 ± 28.6 μmol/kg).

Changes in GAD_{67} or GAD_{65} mRNA Expression. Fig. 3 shows that in frontal cortex GAD_{67} mRNA was significantly increased by about 37% and 26% at 12 and 48 h after diazepam withdrawal, respectively, but the expression of GAD_{65} was not changed. Furthermore, the increase in the expression of GAD_{67} at 12 and 48 h returned to near normal values 96 h after diazepam withdrawal.

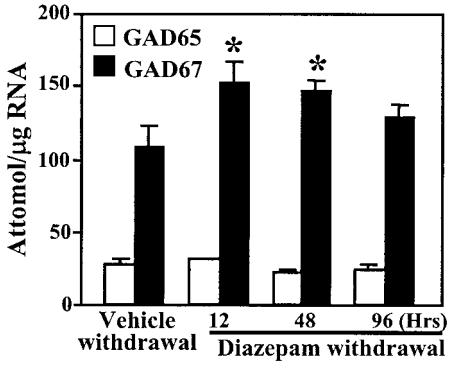

Fig. 3. GAD_{65} and GAD_{67} mRNAs expression in frontal cortex of rats following long-term vehicle- or diazepam-treatment withdrawal. For the diazepam-withdrawn groups the values are mean ± SE of 4–5 animals. For the vehicle-withdrawn group the values were virtually identical at 12, 48, and 96 h after treatment discontinuation and represent mean ± SE of 12 animals. *, $P < 0.05$ compared with the corresponding vehicle-treated rats (ANOVA followed by Duncan's multiple comparison test).

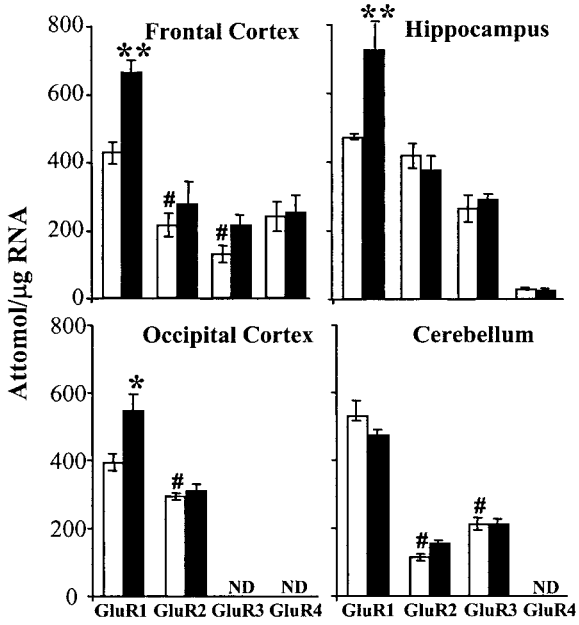

Fig. 4. AMPA GluR receptor subunit mRNAs in rat brain, 96 h after long-term diazepam-treatment (■) or vehicle-treatment (□) withdrawal. Data are mean ± SE of 5–6 animals. *, $P < 0.05$ and **, $P < 0.01$, compared with respective vehicle-treated group; #, $P < 0.01$ when GluR2,3,4 mRNA subunits are compared with GluR 1 mRNA subunit. (ANOVA followed by Duncan's multiple comparison test.) ND, not determined

Time Course Changes in Glutamate Receptor Subunit Expression in Brain of Diazepam-Withdrawn Rats. *AMPA (GluR1–4) and NMDA (NR1) receptor subunit mRNAs.* Fig. 4 demonstrates that in the same animals in which we studied GAD mRNA expression, the levels of GluR1 mRNA were significantly increased in frontal cortex (48%), occipital cortex (38%), and hippocampus (56%), but failed to change in the cerebellum of 96-hr diazepam-withdrawn rats when compared with 96-hr vehicle-withdrawn rats. Notably, the increase in GluR1 mRNA expression detected in the frontal cortex and hippocampus of 96-hr diazepam-withdrawn rats was either not detectable or not statistically significant after 12 or 48 h of diazepam withdrawal (see Fig. 5).

In frontal cortex, GluR2, GluR3, and GluR4 subunit mRNAs expression were between one-half and one-third the level of GluR1 subunit mRNA and their values were similar in diazepam or vehicle-withdrawn rats (Fig. 4). In the hippocampus, the relative abundance of these subunits was different with a rank order of GluR1 = GluR2 > GluR3 > GluR4. Also, in hippocampus there were no changes in the expression of GluR 2, 3, and 4 after 96 h of diazepam withdrawal (Fig. 4). In the same tissue extracts, we measured the expression of NR1 NMDA receptor subunit mRNA. The results shown in Fig. 5 demonstrate that at 96 h after diazepam withdrawal, the content of NR1-NMDA receptor subunit mRNA expression was virtually identical in diazepam and vehicle-withdrawn rats. The levels of NR1-NMDA receptor subunit mRNA also were not modified in the frontal cortex at 12 or 48 h after diazepam withdrawal.

GluR1 subunit protein. (*i*) Western blot: The same brain areas used for GluR1 mRNA analysis were used for a comparative study of GluR1 receptor subunit expression using Western blot (Fig. 6). The antibody for GluR1 immunolabeled a single protein band of 106 kDa, which was increased in intensity by approximately 50% in hippocampus and frontal cortex after 96 h of diazepam withdrawal, but was unchanged after 48-hr withdrawal. All of the values of GluR1 immunoblots were normalized to those of β-actin immunoreactivity. The GluR2 or GluR3

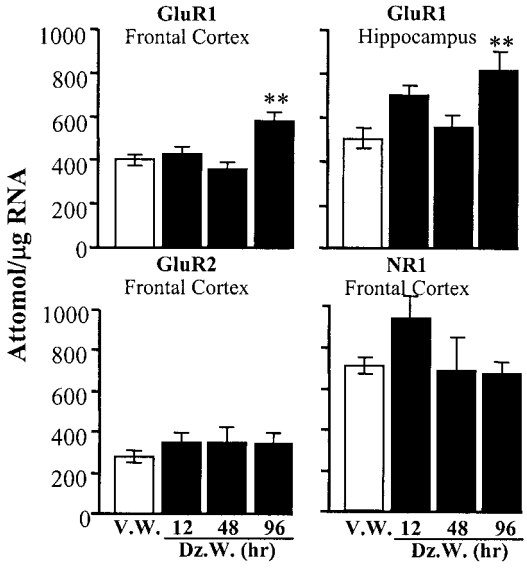

Fig. 5. GluR1, GluR2, and NR1 mRNAs expression in frontal cortex or hippocampus following long-term diazepam-treatment withdrawal (Dz.W.) or vehicle-treatment withdrawal (V.W.). For the Dz.W. groups, values are mean ± SE of 4–5 animals. For the V.W. groups, values were identical at 12, 48, and 96 h after treatment discontinuation; therefore, the data of the three groups were combined and represented as mean ± SE of 12 animals. **, $P < 0.01$ compared with V.W. rats (ANOVA followed by Duncan's multiple comparison test).

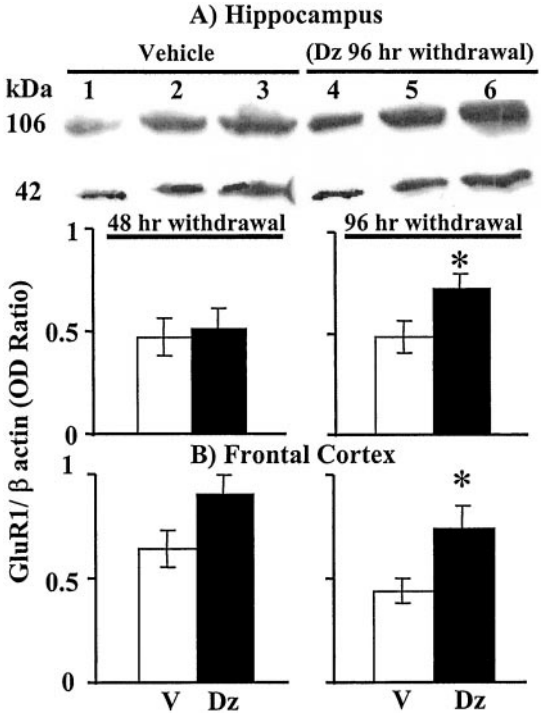

Fig. 6. GluR1 protein expression in hippocampus and frontal cortex following long-term diazepam (Dz)- or vehicle (V)-treatment withdrawal. (*A Upper*) Representative western blot of crude synaptic membrane extracts prepared from vehicle- or diazepam-withdrawn rats. 106-kDa and 42-kDa bands represent GluR1 and β-actin immunoreactivities, respectively. Lanes 1 and 4 were loaded with 10 μg of proteins, lanes 2 and 5 with 20 μg of protein, and lanes 3 and 6 with 40 μg of protein. (*A Lower*) Relative levels of GluR1 receptor subunit expressed as the OD ratio with β-actin. (*B*) Relative levels of GluR1 receptor subunit. Values are the mean ± SE of at least three separate animals. *, $P < 0.05$ when Dz was compared with the respective V group (ANOVA followed by Duncan's multiple comparison test).

subunit proteins were not studied because of the lack of pertinent specific antibodies for each of these two subunits.

(*ii*) GluR1 subunit gold immunolabeling: To perform an immunohistochemical study of GluR1 subunit expression, we used another group of 96-hr diazepam-withdrawn rats. The results of this study show that there was an increase of immunogold GluR1 subunit particle density in selected areas of frontal cortex or hippocampus (Figs. 7 and 8). There was a 30% increase in GluR1 gold-immunolabeling on the pyramidal cell bodies and apical dendrites in layer V of the frontal cortex (Table 1). There was a similar increase in the stratum oriens and in the pyramidal neuron dendritic fields (stratum radiatum and stratum molecularis) of the hippocampus, with a smaller increase (≈19%) in the pyramidal cell somata of hippocampal CA1 region (Table 1).

Discussion

Tolerance and Dependence. *GABA-mediated processes.* Rats treated for 14 days with diazepam and withdrawn for 4 days manifest a decreased number of open-arm entries when tested in an elevated plus-maze. We have interpreted this decrease as a sign of neophobia because it occurred in absence of a change of motor activity (see Fig. 2). Concurrent with neophobia, 4 days after diazepam withdrawal we recorded a 10–15% decrease in body weight (data not shown) and an increased susceptibility to PTZ, a convulsant drug that blocks $GABA_A$ receptor function. These findings are in keeping with our previous report (26) that rats treated for 14 days with the same diazepam schedule used in this study display an increased sensitivity to bicuculline-induced seizures following withdrawal. Previous studies (12–18) have shown that changes of $GABA_A$ receptor subunit expression (decrease of α_1 and γ_2 and increase of α_5) in motor cortex and hippocampus of rodents during tolerance to protracted administration of FAMs cannot be responsible for the withdrawal signs when tolerance remits (see Figs. 1 and 2), because the expression of $GABA_A$ receptor subunits returns to normal (14). Hence, we have studied whether in diazepam-withdrawn rats, a down-regulation of GABAergic transmission could be mediated by a decrease in the expression of the enzyme GAD. There are two types of GAD: GAD_{67} and GAD_{65}. Unlike GAD_{65}, GAD_{67} function is regulated by synthesis and catabolism of the enzyme molecules rather than, as is the case for GAD_{65}, by affinity

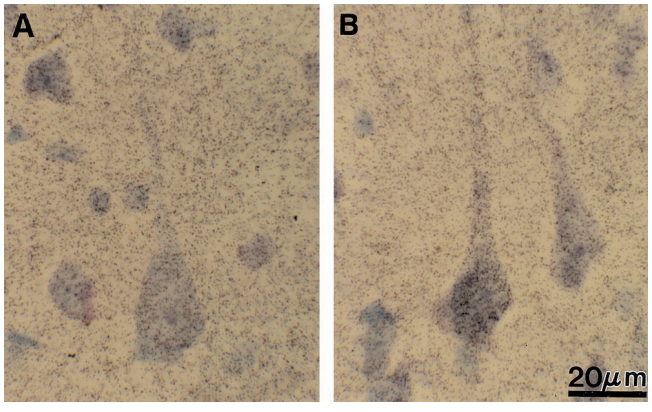

Fig. 7. Photomicrographs of 20-μm sections of layer V of the frontoparietal motor cortex of 96-h vehicle-withdrawn (*A*) or diazepam-withdrawn (*B*) rats. Sections were immunogold labeled for the GluR1 receptor subunit, and counterstained with toluidine blue Nissl stain. Note that in the diazepam-treated rat there is an increase in the expression of GluR1 subunits (black particles) on pyramidal cell bodies and apical dendrites, but not in the surrounding neuropile.

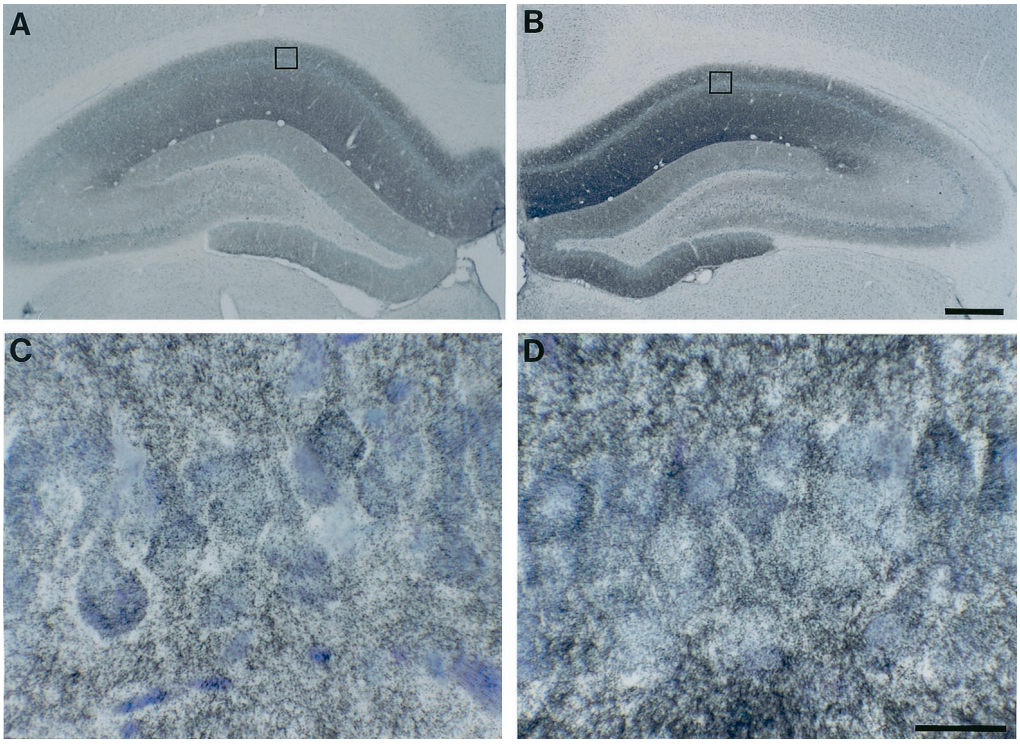

Fig. 8. Photomicrographs of 20-μm sections to the anterior hippocampus of 96 h vehicle-withdrawn (*A* and *C*) or diazepam-withdrawn (*B* and *D*) rats. Staining as in Fig. 7. Note the increased expression of immunogold-labeled GluR1 receptor subunits (black particles) in all layers of the CA1 region of the hippocampus in the diazepam-withdrawn rat. *C* and *D* are higher magnifications of the boxed areas in *A* and *B*, respectively. [Scale bar, 500 μm (*Upper*) and 20 μm (*Lower*).]

changes of the molecule for the cofactor pyridoxal phosphate (30). Our study shows that GAD_{67} and GAD_{65} mRNAs are not decreased after 14-day treatment with diazepam, as initially hypothesized. Actually, the cortical expression of GAD_{67} mRNA is increased by ≈30% at 12 h after the last diazepam treatment. This increase persists for more than 48 h after diazepam withdrawal, but returns to control value 72 h after diazepam discontinuation. The return of GAD_{67} to baseline coincides with the normalization of the α_1, γ_2, and α_5 $GABA_A$ receptor subunit mRNA expressions that were down-regulated during tolerance (14) and with the disappearance of tolerance to the anticonvulsant action of diazepam (see Figs. 1 and 3). Thus, the increase of GAD_{67} expression is time-related to tolerance (compare Figs. 1 and 3) and precedes the expression of withdrawal signs (compare Figs. 2 and 3).

Excitatory amino acid-mediated processes. Steppuhn and Turski (11) and Dunworth and Stephens (23) reported that in mice they could either prevent the expression or block the manifestation of diazepam withdrawal symptoms by injecting AMPA and NMDA receptor antagonists. They hypothesized that a long-term treatment with BZ-RS ligand acting as FAMs, may cause a maximal amplification of GABA-gated Cl^- current intensities, leading to a compensatory enhancement of glutamate receptor function, which they argue tends to minimize the consequences of an

Table 1. Immunogold labeling of GluR1 receptor subunit in frontoparietal motor cortex and hippocampus of rats exposed for 14 days to vehicle (V) or diazepam (D) and withdrawn for 96 hours

	Neuropil*			Pyramidal cells*		
Brain region	V	D	P	V	D	P†
Frontal cortex						
Layer I	54 ± 1.1	48 ± 7.2	0.5	—	—	—
Layer II	41 ± 2.5	44 ± 8.1	0.8	62 ± 2.3	57 ± 7.5	0.5
Layer III	39 ± 1.0	37 ± 3.4	0.7	58 ± 1.6	61 ± 3.6	0.5
Layer IV	48 ± 1.7	49 ± 4.1	0.7	—	—	—
Layer V	37 ± 1.2	45 ± 3.4	0.06	57 ± 3.1	74 ± 3.3	0.005
Layer VI	37 ± 1.5	35 ± 2.6	0.6	58 ± 1.9	63 ± 6.1	0.5
Hippocampus CA1						
Pyramidal cell layer	—	—	—	46 ± 1.5	55 ± 0.7	<0.001
Stratum oriens	52 ± 0.9	74 ± 3.5	<0.001	—	—	—
Stratum radiatum	51 ± 2.1	67 ± 2.9	<0.002	—	—	—
Stratum molecularis	58 ± 1.3	77 ± 2.6	<0.001	—	—	—

*Mean ± S.E.M. of the number of immunogold particles per 100 μm^2; five animals/group.
†*P* = Statistical difference determined by ANOVA followed by Fisher's least significant difference test.

up-regulation of GABAergic tone elicited by BZ-RS ligands. The present report shows that in rats withdrawn from diazepam for 96 h (when signs of dependence appear), there is an increased expression of GluR1 subunit mRNA and cognate proteins in the cortex and hippocampus, but not in cerebellum. Moreover, by immunohistochemistry we established that in cortex, the increase in GluR1 immunoreactivity is mostly localized to the pyramidal cell somata and apical dendrites. In the hippocampus, the increase in GluR1 expression is predominantly in the pyramidal cell dendritic field and therefore probably in dendritic spine synapses of principal neurons, rather than on the cell bodies and dendrites of the interneurons.

Because the increase of GluR1 subunit mRNA and protein expression coincides with the termination of GAD_{67} up-regulation, the increase in the expression of GluR1 in frontal cortex and hippocampus may be revealing a molecular mechanism underlying the expression of withdrawal signs. The quantitative RT-PCR assay used in our experiments to measure GluR1 mRNA subunit expression does not differentiate between Flop and Flip isoforms of the different AMPA receptor subunits. However, as indicated by several studies that we have confirmed (14), the adult mammalian brain expresses mostly the Flop isoform of these subunits. In preliminary experiments, the Flop/Flip ratio for the mRNA (9:1) of the GluR1 subunit was not found to be modified by 14-day treatment with diazepam or by diazepam withdrawal (data not shown).

It is interesting to note, however, that the increase of GluR1 subunit mRNA in cortex and hippocampus does not generalize to other glutamate receptor subunit mRNAs; specifically, it occurs in the absence of changes in the mRNAs encoding for GluR2, GluR3, GluR4, or NR1-NMDA receptor subunits. The NR1 subunit mRNA was measured with a set of PCR primers that comprise all of the NR1 transcripts. Because the NR1 subunit is required to form any functional NMDA receptor subtype and its pattern of distribution is rather ubiquitous throughout the brain, this would suggest that an alteration of NMDA receptor function (11), after an abrupt discontinuation of diazepam treatment, may not depend on a primary increase in the NMDA receptor density. However, our experiments do not rule out a change in NMDA receptor subunit assembly due to changes in NR2 subunit expression in discrete brain nuclei or subcellular compartments.

Significance of Glutamate and $GABA_A$ Receptor Subunit Expression in Dendritic Compartments. Despite the existence of a significant correlation between the biochemical (mRNA) and the immunohistochemical (protein) data for GluR1 subunit, one question that remains to be addressed is how these overexpressed GluR1 subunits integrate with other GluR subunits in diazepam-dependent rats, and whether they are assembled with the same stoichiometry found in AMPA receptors of naive animals. For example, it has been demonstrated that long-lasting changes of synaptic AMPA receptor density that occur during the induction and expression of LTP or LTD require both the delivery of receptor subunits to the dendritic spine membranes and an increased capacity of the same membranes to bind and immobilize these subunits in properly assembled receptors to produce an increased density of functional receptors (31). Thus, the significance of measurements of receptor subunit mRNA or protein expression carried out in brain homogenates must be interpreted with caution because this measurement fails to detect whether these changes occur in specific neuronal compartments such as somata, dendrites, spines, or even nonuniformly in specific dendritic compartments. It is now established that it is in the spines that a modification of a relatively small pool of resident mRNAs encoding for receptor subunit proteins that are translated locally may have important consequences in changing the strength of synaptic function during LTP or LDP (32, 33). Recent findings show that mRNAs for $GABA_A$, AMPA, or NMDA receptor subunits are resident in dendrites in proximity of spines (33), which express all of the machinery for mRNA translation (34). This necessitates the comparison in vehicle and diazepam-treated animals, of whether mRNAs encoding for GABA and glutamate ionotropic receptors are increased selectively in the short (dendrites and spines) or long loop (somata) of pyramidal neurons. To this end, the dendritic and somatic pools of $GABA_A$, AMPA, and NMDA receptor subunit mRNAs should be determined by confocal and electron microscopy. In addition, an investigation is needed into changes in the expression of postsynaptic density-95, discs large end ZO-1 (PDZ) domain-containing scaffolding proteins such as glutamate-receptor-interacting protein (GRIP), PDS-95kDa, gephyrin (35) and $GABA_A$receptor-associated protein (GABARAP) (36), which are known to participate in the stabilization and remodeling of synapses and to enhance post synaptic clustering and activity of AMPA, NMDA and $GABA_A$ receptors, respectively.

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