Behavioral/Systems/Cognitive

Requirement of α_5-GABA$_A$ Receptors for the Development of Tolerance to the Sedative Action of Diazepam in Mice

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Despite its pharmacological relevance, the mechanism of the development of tolerance to the action of benzodiazepines is essentially unknown. The acute sedative action of diazepam is mediated via α_1-GABA$_A$ receptors. Therefore, we tested whether chronic activation of these receptors by diazepam is sufficient to induce tolerance to its sedative action. Knock-in mice, in which the α_1-, α_2-, α_3-, or α_5-GABA$_A$ receptors had been rendered insensitive to diazepam by histidine–arginine point mutation, were chronically treated with diazepam (8 d; 15 mg · kg^{-1} · d^{-1}) and tested for motor activity. Wild-type, α_2(H101R), and α_3(H126R) mice showed a robust diminution of the motor-depressant drug action. In contrast, α_5(H105R) mice failed to display any sedative tolerance. α_1(H101R) mice showed no alteration of motor activity with chronic diazepam treatment. Autoradiography with [^{3}H]flumazenil revealed no change in benzodiazepine binding sites. However, a decrease in α_5-subunit radioligand binding was detected selectively in the dentate gyrus with specific ligands. This alteration was observed only in diazepam-tolerant animals, indicating that the manifestation of tolerance to the sedative action of diazepam is associated with a downregulation of α_5-GABA$_A$ receptors in the dentate gyrus. Thus, the chronic activation of α_5-GABA$_A$ receptors is crucial for the normal development of sedative tolerance to diazepam, which manifests itself in conjunction with α_1-GABA$_A$ receptors.

Key words: **diazepam; GABA$_A$ receptor; tolerance; motor activity; dentate gyrus; knock-in mice**

Introduction

Loss of sedative efficacy of diazepam with chronic treatment has been proposed to result from the development of adaptive processes counteracting the repeated enhancement by the benzodiazepine of GABA$_A$ receptor-mediated inhibitory neurotransmission (Steppuhn and Turski, 1993; File and Fernandes, 1994; Fernandes et al., 1996; Marin et al., 1996, 1999; Perez et al., 2003). Functional alterations of GABA$_A$ receptors have frequently been reported after various chronic treatment regimens with diazepam (Hutchinson et al., 1996; Itier et al., 1996; Primus et al., 1996; Ali and Olsen, 2001; Costa et al., 2001; Bateson, 2002). Uncoupling of the allosteric interaction between the benzodiazepine binding site and the GABA site, probably linked to GABA$_A$ receptor internalization, has been proposed as a correlate of diazepam tolerance (Hutchinson et al., 1996; Itier et al., 1996; Primus et al., 1996; Ali and Olsen, 2001; Costa et al., 2001). Furthermore, subtle changes in the expression of GABA$_A$ receptor subunits were described notably in the cerebral cortex and hippocampus (Wu et al., 1994; Impagnatiello et al., 1996; Pesold et al., 1997; Arnot et al., 2001). In particular, a selective decrease in the expression of genes encoding the α_1- and γ_2-subunits in dendrites and spines of cortical pyramidal cells has been associated with tolerance to the anticonvulsant action of diazepam (Costa et al., 2002). In addition, α_5-GABA$_A$ receptors were affected, as shown by an increase in α_5-subunit mRNA in the frontoparietal cortex or a reduced radioligand binding in the hippocampus after 2 or 3 weeks of diazepam administration (Impagnatiello et al., 1996; Pesold et al., 1997; Li et al., 2000).

The recognition of distinct pharmacological functions of GABA$_A$ receptor subtypes opened new avenues to investigate the mechanisms of tolerance. Using a histidine-to-arginine point mutation strategy that selectively abolishes diazepam binding to GABA$_A$ receptors containing the α_1-, α_2-, α_3-, or α_5-subunit *in vivo*, it has been shown that α_1-GABA$_A$ receptors mediate the acute sedative (Rudolph et al., 1999; McKernan et al., 2000) and anticonvulsant (Rudolph et al., 1999) properties of diazepam, whereas α_2-GABA$_A$ receptors are the substrate for anxiolytic (Löw et al., 2000) and muscle-relaxant activity, the latter also requiring α_3- and α_5-GABA$_A$ receptors (Crestani et al., 2001, 2002). In view of such functional receptor specificity, we tested whether tolerance to a particular effect of diazepam is mediated via the same receptor subtype involved in the acute effect or whether additional GABA$_A$ receptor subtypes are required for neuronal plasticity leading to the development of tolerance with chronic diazepam treatment.

In the present study, the contribution of specific GABA$_A$ re-

Received March 23, 2004; revised June 1, 2004; accepted June 16, 2004.

This work was supported by the Swiss National Science Foundation. We thank K. Löw for the generation of the α_2(H101R) mice, D. Benke for help with autoradiography, and C. Sidler, H. Pocchetti, and E. Calame for technical assistance.

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DOI:10.1523/JNEUROSCI.1067-04.2004

 0270-6474/04/246785-06$15.00/0

ceptor subtypes in the development of tolerance to the motor-depressant action of diazepam was examined using wild-type and histidine–arginine point-mutated mice. Potential changes in benzodiazepine binding sites were analyzed by autoradiography.

Materials and Methods

Animals and drugs. Nine- to 11-week-old female wild-type, α_1(H101R), α_2(H101R), α_3(H123R), and α_5(H105R) mice (>10 backcrosses to 129/SvJ background) were used (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2002). The sensitivity of $GABA_A$ receptors to diazepam is conferred by the presence of a histidine residue at a conserved position in the extracellular portions of the α subunits (α1-H101, α2-H101, α3-H126, α-H105). The histidine–arginine substitution in the $GABA_A$ receptor α_1-, α_2-, α_3-, or α_5-subunit results in a marked reduction of the binding affinity of the corresponding $GABA_A$ receptor subtype to diazepam, as shown on recombinant receptors (Benson et al., 1998). Thus, each point-mutated mouse line possesses a particular $GABA_A$ receptor subtype insensitive to allosteric modulation by diazepam, although its response to GABA is essentially preserved. Females were preferred to males because of the prominent intramale aggressive behavior inherent to the strain used as genetic background. They were reared in group-housed cages in the testing room under reversed 12 hr light/dark conditions. Treatments and behavioral testing were performed during the dark phase. The Cantonal Veterinary Office of Zürich approved all experimental procedures. Diazepam was from Hoffmann-La Roche (Basel, Switzerland).

Induction of sedative tolerance. Mice were subjected to daily injections of diazepam (10 mg/kg at 10 A.M. and 5 mg/kg at 4 P.M., i.p.) for 8 d in the home cage. On day 9, half of the mice were given vehicle (Diaz-Veh) and the other half were given the morning dose of 10 mg/kg diazepam as the test dose. Control animals, which received the vehicle (0.3% Tween 80–saline solution) as chronic treatment, were distributed in two groups. One group was treated with vehicle (Veh–Veh), and the other group was treated with 10 mg/kg diazepam. A fifth group of mice, which served as control for the effects of repeated injections, received a single diazepam (10 mg/kg) injection as the test dose. Animals were left undisturbed to experience the drug effects in the home cage for 30 min. At the end of this period, they were placed in individual circular alleys (Imetronic, Pessac, France) for motor-activity assessment, measured as the number of photocell interruptions during a 10 min period. The term test dose was used to indicate the association of the last diazepam morning injection of the chronic treatment with the behavioral assessment. The terms sedation and sedative, as defined by Katzung (1995), indicate the drug-induced decrease in the animal's spontaneous activity. Measurement of motor activity in rodents represents a standard behavioral assay for testing the sedative potential of drugs (Vogel, 2002).

Quantitative receptor autoradiography. A second series of animals received the same chronic treatment regimen with either vehicle or diazepam. On day 9, tolerance to the motor-depressant action of 10 mg/kg diazepam was tested, and the mice were killed by decapitation 5 hr thereafter. Binding assays using [^{3}H]flumazenil (PerkinElmer, Boston, MA) or two ligands with a preferential affinity for the α_5-subunit (Quirk et al., 1996; Skolnick et al., 1997), [^{3}H]RY80 (PerkinElmer) and [^{3}H]L655708 (Amersham Biosciences, Otelfingen, Switzerland), were performed on transverse 12 μm brain cryosections (Fritschy et al., 1997). Briefly, after a 1 hr incubation with 12 nM [^{3}H]flumazenil or a 2 nM concentration of either [^{3}H]RY80 or [^{3}H]L655708 in 50 mM Tris–Cl at pH 7.5, the sections were exposed to a tritium-sensitive phosphor screen (Packard Cyclone Storage Phosphor System; Packard, Meridian, CT) for 2 or 8 d, respectively. Adding 10 μM clonazepam assessed unspecific labeling. The screens were digitized with a Packard Cyclone Scanner, and labeling intensities were measured in the motor cortex, striatum, nucleus accumbens, and hippocampal formation (CA1, CA3 stratum oriens–pyramidale, and dentate gyrus) of both hemispheres. At the concentrations used, both [^{3}H]RY80 and [^{3}H]L655708 have been reported to saturate α_5-subunit binding sites with high affinity in hippocampal membranes (Quirk et al., 1996; Skolnick et al., 1997; Sur et al., 1999).

Statistics. Results, expressed as mean ± SE (or SD for binding studies), were analyzed using nonparametric Kruskal–Wallis analysis and, whenever appropriate, Mann–Whitney tests for *post hoc* mean comparisons.

Results

Tolerance to the sedative action of diazepam in point-mutated mice

To identify the diazepam-sensitive $GABA_A$ receptor subtypes implicated in sedative tolerance, we examined the potential of histidine–arginine point-mutated mice to develop tolerance against the motor-depressant action of diazepam during the course of a chronic drug-treatment regimen. We focused on this behavioral effect for two main reasons. First, the effectiveness of diazepam in decreasing motor activity in rodents is considered as a valid behavioral manifestation of its sedative properties (Vogel, 2002). Second, this drug effect is exclusively mediated by α_1-$GABA_A$ receptors, because it is abolished in α_1(H101R) mice but unaltered in α_2(H101R), α_3(H126R), and α_5(H105R) mice (Rudolph et al., 1999; Löw et al., 2000; McKernan et al., 2000; Crestani et al., 2002).

Sedative tolerance to diazepam (i.e., the diminution of its motor-depressant action during chronic treatment) was first analyzed using wild-type and α_1(H101R) mice. The different treatment conditions affected motor activity differently in wild-type mice ($H = 22.958$; $p < 0.001$; $n = 6$–7 mice per group). In wild-type mice chronically treated with vehicle, the administration of 10 mg/kg diazepam was followed by a marked decrease in motor activity ($p < 0.01$ compared with Veh–Veh) (Fig. 1*a*). This effect was comparable with that seen in mice that received a single acute injection of diazepam. Mice chronically treated with diazepam did not show a reduction in motor activity in response to the test dose of diazepam and were indistinguishable from mice challenged with vehicle or from mice chronically treated with vehicle only (Fig. 1*a*). In α_1(H101R) mice, neither a single acute injection of diazepam (10 mg/kg) nor the chronic diazepam treatment altered the level of motor activity compared with the chronic vehicle treatment, as revealed by the lack of statistical significance of the overall analysis of the effects of the different drug-treatment conditions ($H = 5.914$; not significant; $n = 6$ mice per group) (Fig. 1*b*). The increased motor activity seen in response to diazepam in α_1(H101R) mice chronically treated with vehicle achieved significance only when compared, in a separate two-mean comparison, with the effect of the vehicle test injection ($p < 0.01$; Mann–Whitney test) but not when compared with that induced by a single acute diazepam injection (Fig. 1*b*).

To characterize the role of diazepam-sensitive $GABA_A$ receptor subtypes other than those containing the α_1-subunit in sedative tolerance, α_2(H101R), α_3(H126R), and α_5(H105R) mice were subjected to the same chronic diazepam treatment regimen. There was a significant overall effect of the different treatment conditions on motor activity in all three mutant lines [α_2(H101R), $H = 14.942$, $p < 0.001$, $n = 8$ mice per group; α_3(H126R), $H = 12.194$, $p < 0.002$, $n = 8$ mice per group; α_5(H105R), $H = 12.005$, $p < 0.002$, $n = 8$ mice per group]. Diazepam (10 mg/kg) failed to decrease motor activity levels in α_2(H101R) and α_3(H126R) mice chronically treated with diazepam, but it induced sedation in those mutants chronically treated with vehicle ($p < 0.01$ compared with the respective Veh–Veh groups) (Fig. 1*c*). However, in α_5(H105R) mice, the same test dose of diazepam was equally effective in depressing motor activity in animals chronically treated with either vehicle or diazepam ($p < 0.01$ compared with Veh–Veh) (Fig. 1*c*). Thus, tolerance to the motor-depressant action of diazepam developed to the same extent in wild-type, α_2(H101R), and α_3(H126R) mice within 8 d of chronic drug administration, but was not seen in α_5(H105R) mice. Furthermore, the same chronic diazepam treatment regimen did not alter motor activity in α_1(H101R) mice.

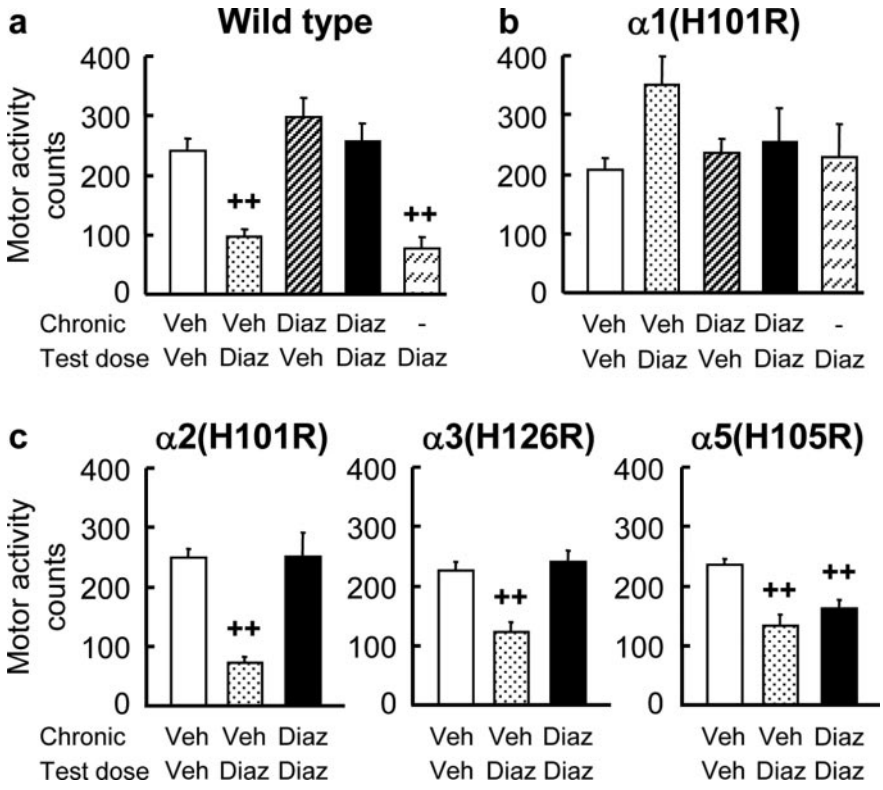

Figure 1. Motor activity in wild-type and point-mutated mice subjected to an 8 d chronic diazepam treatment regimen (15 mg · kg $^{-1}$ · d $^{-1}$). *a*, In wild-type mice, the test dose of diazepam (10 mg/kg) was equally effective in decreasing motor activity when given either acutely or 18 hr after a chronic vehicle treatment. Mice chronically treated with diazepam showed levels of motor activity similar to those of mice chronically treated with vehicle in response to either diazepam or vehicle ($H = 22.958$; $p < 0.001$; $n = 6–7$ mice per group). *b*, In α_1(H101R) mice, there was no overall effect of the different treatment conditions on motor activity ($H = 5.914$; not significant; $n = 6$ mice per group). However, an increased motor activity was seen in animals chronically treated with vehicle in response to diazepam ($p < 0.01$ compared with Veh–Veh; Mann–Whitney test). *c*, The test dose of diazepam decreased motor activity in animals chronically treated with the vehicle but not in α_2(H101R) ($H = 14.942$; $p < 0.001$; $n = 8$ mice per group) and α_3(H126R) ($H = 12.194$; $p < 0.002$; $n = 8$ mice per group) mice treated with diazepam. In α_5(H105R) mice, the same diazepam test dose depressed motor activity in animals chronically treated with either vehicle or diazepam ($H = 12.005$; $p < 0.002$; $n = 8$ mice per group). Results are given as means ± SE. $^{++}p < 0.01$ versus Veh–Veh; Mann–Whitney test.

Chronic treatment / Test dose	Wild type Veh Veh	Wild type Veh Diaz	Wild type Diaz Diaz	α1(H101R) Veh Veh	α1(H101R) Veh Diaz	α1(H101R) Diaz Diaz	α2(H101R) Veh Veh	α2(H101R) Veh Diaz	α2(H101R) Diaz Diaz
Motor cortex	183.8±4.8	182.0±2.9	199.6±11.3	61.3±2.0	64.1±1.0	56.3±2.1	154.2±2.8	155.2±5.1	152.3±4.2
Striatum	74.1±2.9	72.0±3.1	76.0±5.4	43.8±1.9	43.5±1.9	39.4±1.0	47.6±1.7	45.3±1.7	43.9±1.6
N.Accumbens	61.4±2.5	61.2±1.6	59.1±4.3	68.6±1.7	72.4±5.1	66.3±1.8	51.2±2.8	47.9±2.0	46.1±2.2
Hippocampus	150.1±5.5	142.0±7.4	140.8±9.2	94.7±4.6	95.1±6.0	88.4±4.1	110.9±12.1	110.5±10.0	115.1±8.3

Figure 2. Autoradiography of [^{3}H]flumazenil binding after cessation of an 8 d treatment regimen with vehicle or diazepam (15 mg · kg $^{-1}$ · d $^{-1}$) and administration of the test dose of diazepam (10 mg/kg) in wild-type, α_1(H101R), and α_2(H101R) mice. Regardless of the genotype, the chronic diazepam treatment did not alter [^{3}H]flumazenil binding compared with chronic vehicle or acute diazepam, as quantified for three regions involved in motor control and for the hippocampal formation. Standards and the differential [^{3}H]flumazenil binding profile related to the point mutation are also presented in representative transverse sections from animals chronically treated with vehicle. The different binding levels in the mutant mice reflect the loss of diazepam binding to the mutated subunit. Results are expressed in mean ± SD nanocuries per milligram of protein; $n = 4–7$ mice per group.

Brain autoradiography of α_5-GABA$_A$ receptor binding sites

To assess whether the expression of sedative tolerance to diazepam would be associated with alterations in GABA$_A$ receptor binding sites, we analyzed benzodiazepine binding autoradiographically with [^{3}H]flumazenil in wild-type, α_1(H101R), and α_2(H101R) mice. The levels of [^{3}H]flumazenil binding differed across genotypes as a result of the presence of the respective point mutation (Fig. 2). However, no alteration in relation to the chronic drug-treatment regimen and the behavioral testing was detected, in particular in forebrain regions involved in the control of motor activity, including the primary motor cortex, striatum, and nucleus accumbens, as well as in the hippocampal formation (Fig. 2). Thus, there was no evidence for a general GABA$_A$ receptor downregulation.

To specifically analyze the role of α_5-GABA$_A$ receptors, two selective α_5-subunit ligands, [^{3}H]RY80 and [^{3}H]L655708, were used in the autoradiographic analysis. In wild-type mice, the levels of [^{3}H]RY80 binding were differentially affected by the three treatment conditions in the dentate gyrus ($H = 14.769$; $p < 0.001$; $n = 7–8$ mice per group) (Fig. 3*a*). A significantly lower [^{3}H]RY80 binding level (−13.6% relative to Veh–Veh) was observed in diazepam-tolerant animals ($p < 0.01$ compared with Veh–Veh and Veh–Diaz). A similar effect of the chronic diazepam treatment was confirmed using [^{3}H]L655708 as the radioligand ($H = 10.903$; $p < 0.004$; $n = 6–7$ mice per group) (Fig. 3*b*). The [^{3}H]L655708 binding level was significantly reduced (14.7% relative to Veh–Veh) only in tolerant mice ($p < 0.01$ compared with Veh–Veh and Veh–Diaz). In contrast, the effects of the three treatment conditions on [^{3}H]RY80 and [^{3}H]L655708 binding levels were comparable in the hippocampal CA1 area ([^{3}H]RY80, $H = 1.563$, not significant, $n = 5–6$ mice per group; [^{3}H]L655708, $H = 3.661$, not significant, $n = 6–7$ mice per group) as well as in the CA3 area ([^{3}H]RY80, $H = 4.662$, not significant, $n = 5–6$ mice per group; [^{3}H]L655708, $H = 1.988$, not significant, $n = 6–7$ mice per group) (data not shown).

[^{3}H]RY80 binding was also analyzed in α_1(H101R) and α_2(H101R) mice subjected to the same chronic drug treatment and behavioral testing. In α_2(H101R) mice, as in wild-type mice, the chronic administration of diazepam was accompanied by sedative tolerance (data not shown) and by a decrease in [^{3}H]RY80 binding levels in the dentate gyrus (−12.5% relative to Veh–Veh; $p < 0.01$) ($H = 9.231$; $p < 0.009$; $n = 5–6$ mice per group) (Fig. 3*c*). The α_1(H101R) mice displayed similar levels of [^{3}H]RY80 binding regardless of the treatment conditions ($H = 2.788$; not significant; $n = 5–6$ mice per group) (Fig. 3*d*). Thus, the manifestation of tolerance to the motor-depressant action of

diazepam was accompanied by an apparent reduction of α_5-GABA$_A$ receptor binding selectively in the dentate gyrus, as shown for wild-type and α_2(H101R) mice. This reduction of α_5-subunit binding did not occur in α_1(H101R) mice subjected to the same chronic diazepam treatment.

Discussion

The present results point to a critical role of α_5-GABA$_A$ receptors, in conjunction with α_1-GABA$_A$ receptors, in the development of tolerance against the sedative action of diazepam. Interaction of diazepam with α_5-GABA$_A$ receptors appears to be a prerequisite for the normal development of tolerance against its motor-depressant action. This is shown by the retained capacity of diazepam (10 mg/kg) to efficiently reduce motor activity in α_5(H105R) mice, which possess diazepam-insensitive α_5-GABA$_A$ receptors, at least within the limits of our chronic drug-treatment regimen (Fig. 1*c*). This motor effect cannot be attributed to a retained myorelaxant action of diazepam, because we have shown that these mutants, as well as the α_2(H101R) mice, do not express the acute myorelaxant action of diazepam in the range of doses used here for the chronic treatment (Crestani et al., 2001, 2002). However, α_2(H101R) and α_3(H126R) mice developed sedative tolerance to diazepam to the same extent as wild-type mice (Fig. 1*c*), indicating that diazepam-sensitive α_2- or α_3-GABA$_A$ receptors are not essential for this effect. In rodents, tolerance to the motor-depressant action of diazepam develops rapidly, within 3–5 d of chronic administration (Steppuhn and Turski, 1993; File and Fernandes, 1994; Marin et al., 1996). We designed a chronic treatment protocol, using high doses of diazepam over a period of 9 d, to induce a robust sedative tolerance in our control wild-type mice. Nevertheless, we cannot rule out the possibility of a delayed occurrence of sedative tolerance (i.e., after a longer chronic diazepam treatment) in α_5(H105R) mice. Conversely, the absence of a motor-depressant drug effect in wild-type, α_2(H101R), and α_3(H126R) mice with chronic treatment could reflect a rightward shift in the dose–response curve of diazepam, with higher doses of diazepam (>10 mg/kg) producing sedation. However, this appears improbable, especially with regard to our test conditions. Motor activity was measured 30 min after the last diazepam injection of the chronic treatment and not after a period of drug withdrawal, as is often the case in the literature, to assess the retained sedative drug efficacy at a particular time of the chronic diazepam treatment regimen. To our knowledge, only the study by Perrault et al. (1993), using the anticonvulsant action of diazepam as endpoint, described a rightward and downward shift in the dose–response curve of diazepam, but only when the test dose was given 42 hr after termination of a 10 d chronic treatment regimen (5 mg/kg, p.o., twice daily). This shift was attributed to the rapid development of a hypersensitivity of the mice to the convulsant drug in relation to the diazepam withdrawal experience. A significant residual anticonvulsant action of diazepam was seen in chronically treated animals when the convulsant drug was administered 6 hr after the last diazepam dose (Perrault et al., 1993).

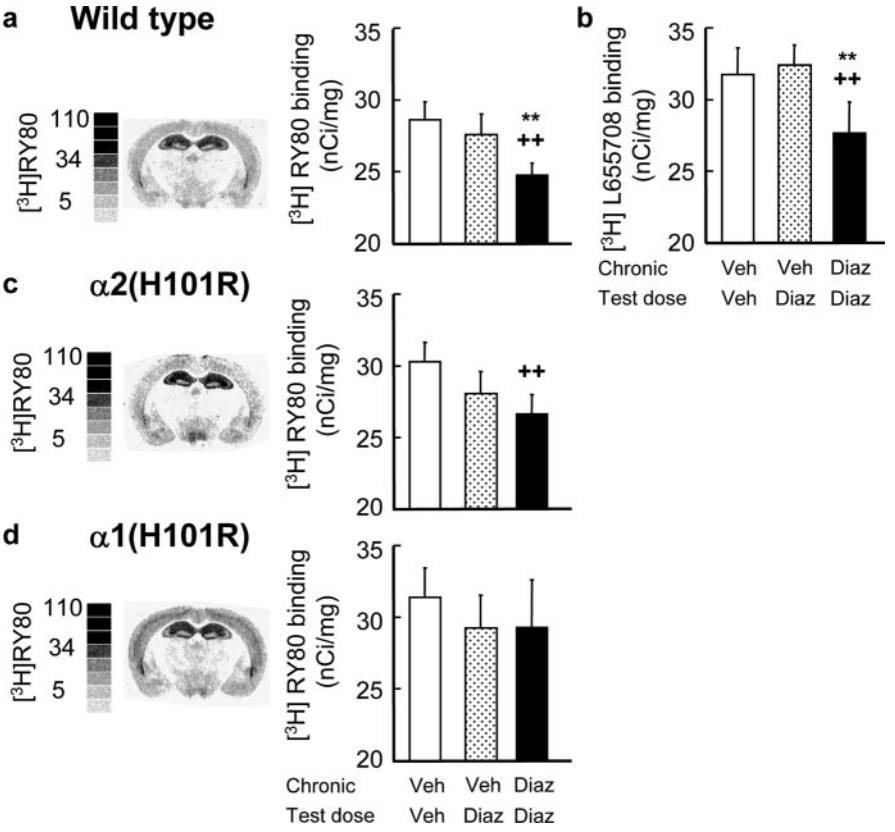

Figure 3. Alteration of α_5-GABA$_A$ receptor binding in dentate gyrus after cessation of an 8 d treatment regimen with vehicle or diazepam (15 mg • kg^{-1} • d^{-1}) assessed autoradiographically with [^{3}H]RY80 and [^{3}H]L655708 in wild-type mice (*a*, *b*) and with [^{3}H]RY80 in α_2(H101R) (*c*) and α_1(H101R) (*d*) mice. Standards and representative transverse sections from mice chronically treated with vehicle are shown. *a*, [^{3}H]RY80 binding levels were decreased in wild-type mice chronically treated with diazepam. *b*, A similar decrease in [^{3}H]L655708 binding level was observed in the same wild-type mice. *c*, In α_2(H101R) mice, only animals chronically treated with diazepam showed a significant reduction of [^{3}H]RY80 binding. *d*, In α_1(H101R) mice, no alteration in [^{3}H]RY80 binding was seen, regardless of the chronic drug treatment. Results are given as mean ± SD nanocuries per milligram of protein. $^{++}p < 0.01$ versus Veh–Veh; $^{**}p < 0.01$ versus Veh–Diaz; Mann–Whitney test.

The diminution of motor activity seen in α_5(H105R) mice with chronic diazepam treatment also reveals that α_1-GABA$_A$ receptors remain responsive during the course of the chronic diazepam treatment, recurrently mediating sedation in these mutants. Therefore, sedative tolerance does not appear to be attributable to a reduction of the motor-depressant efficacy of diazepam, thus confirming a previous report (Bourin et al., 1992). Rather, its manifestation might be secondary to the development of an α_5-GABA$_A$ receptor-dependent response to chronic diazepam that would oppose its motor-depressant action. In α_1(H101R) mice, which do not display the sedative drug action, chronic interaction of diazepam with GABA$_A$ receptors other than those containing the α_1 subunit was not associated with a change in motor activity (Fig. 1*b*). This result argues against an oppositional α_1-GABA$_A$ receptor-independent mechanism, which would counterbalance and thus mask the sedative drug action. α_1- and α_5-GABA$_A$ receptors appear to be the specific molecular substrates contributing in a competitive manner to the chronic effects of diazepam on motor activity. Chronic interaction of diazepam with α_1-GABA$_A$ receptors results in a recurrent motor-depressant action while the concurrent interaction with α_5-GABA$_A$ receptors is essential for the behavioral manifestation

of tolerance to this effect. This result is in line with reports indicating that benzodiazepine site ligands that do not interact with α_5-GABA$_A$ receptors, such as zolpidem, show little or no evidence for sedative tolerance in rodents and fail to alter α_5-subunit levels (Zivkovic et al., 1994; Costa and Guidotti, 1996; Holt et al., 1997).

The α_5-GABA$_A$ receptors of the dentate gyrus appear to be a specific target for adaptive changes associated with sedative tolerance to diazepam. Indeed, a downregulation of α_5-GABA$_A$ receptors, as assessed with two selective α_5-subunit radioligands used at saturating concentrations, was observed only in wild-type and α_2(H101R) mice tolerant to the motor-depressant action of diazepam (Fig. 3*a–c*). This reduction of receptors was restricted to the dentate gyrus. No change in α_5-subunit radioligand binding levels was detected in the CA1 and CA3 areas of these animals. Within the limits of statistical power, a decreased binding of α_5-subunit-specific radioligands in diazepam-tolerant animals could be resolved only in the hippocampal formation. We admit that the chronic diazepam treatment regimen used here can give rise to alterations in α_5-GABA$_A$ receptors in other brain regions. However, the unchanged levels of [^{3}H]flumazenil binding in forebrain regions involved in control of motor activity argues against a general downregulation of GABA$_A$ receptors and against a regionally specific loss of another major GABA$_A$ receptor subtype after chronic diazepam treatment. This observation is in concordance with other reports that flumazenil binding sites remain unaltered after chronic benzodiazepine treatment (Hutchinson et al., 1996; Costa et al., 2001; Bateson, 2002). The absence of alteration in α_5-subunit radioligand binding in α_1(H101R) mice chronically treated with diazepam (Fig. 3*d*) further indicates that occurrence of α_5-GABA$_A$ receptor downregulation in the dentate gyrus closely depends on the chronic interaction of the drug with α_1-GABA$_A$ receptors but not with GABA$_A$ receptors containing the α_2-, α_3-, or α_5-subunit.

α_5-GABA$_A$ receptors constitute a minor population of diazepam-sensitive GABA$_A$ receptors. They are found mainly in the hippocampal formation, olfactory bulb granule cell layer, and spinal cord dorsal horn and in lower amounts in the cerebral cortex and hypothalamus (Crestani et al., 2002). We have reported previously that the H105R point mutation in the α_5 subunit gene resulted in a reduction of α_5-GABA$_A$ receptors in the dendritic layers of the hippocampal CA1 and CA3 areas with no change in the dentate gyrus (Crestani et al., 2002). Although this specific deficit of receptors in α_5(H105R) mice mimics to some extent the molecular changes seen in the dentate gyrus of diazepam-tolerant animals, it does not interfere with the expression of the motor-depressant action of diazepam when given acutely (Crestani et al., 2002) or chronically (Fig. 1*c*). This is in keeping with the lack of alteration in the expression pattern of the other major GABA$_A$ receptor subunits, notably the α_1-subunit, in α_5(H105R) mice (Crestani et al., 2002) as well as in mice with a complete loss of hippocampal α_5-GABA$_A$ receptors (Collinson et al., 2002). Thus, it is not a reduction of α_5-GABA$_A$ receptors per se, but rather the selective localization in the dentate gyrus, which appears to be associated with the expression of sedative tolerance to diazepam. Likewise, the induction of long-term potentiation in hippocampal pyramidal cells is unaltered in α_5(H105R) mice (Crestani et al., 2002), whereas tolerance to the motor-depressant action of diazepam is associated with an increased synaptic plasticity in the rat dentate gyrus (Marin et al., 1996). Therefore, the failure of α_5(H105R) mice to manifest sedative tolerance, despite their partial deficit in hippocampal α_5-GABA$_A$ receptors, strengthens the hypothesis that diazepam binding to α_5-GABA$_A$ receptors is a key mechanism underlying the robust diminution of its sedative efficacy with chronic treatment. This is striking, because these receptors are mainly extrasynaptic and mediate tonic inhibition in hippocampal CA1 pyramidal cells (Crestani et al., 2002; Caraiscos et al., 2004). The association of changes in α_5-subunit binding sites, mRNA, or protein levels with tolerance to diazepam has been reported previously concerning its anticonvulsant properties (Wu et al., 1994; Impagnatiello et al., 1996; Pesold et al., 1997; Li et al., 2000). Here, we demonstrate that the decrease in α_5-subunit binding in the dentate gyrus depends on the chronic activation by diazepam of α_1-GABA$_A$ receptors, which primarily produce phasic inhibition in the brain. This is in keeping with the reported high plasticity in the expression of hippocampal extrasynaptic α_5-GABA$_A$ receptors in response to intense synaptic activity (Houser and Esclapez, 2003).

In conclusion, we propose that the manifestation of tolerance to the motor-depressant action of diazepam depends on the chronic activation of two competitive mechanisms orchestrated by α_1- and α_5-GABA$_A$ receptors, respectively. Chronic drug interaction with α_1-GABA$_A$ receptors results in a persistent augmentation of phasic inhibition in the forebrain areas involved in motor control, mediating motor depression. This recurrent increased phasic signaling would alter the weight of the tonic inhibition produced by the simultaneous drug activation of extrasynaptic α_5-GABA$_A$ receptors in the hippocampal formation. The α_1-GABA$_A$ receptor-dependent change in inhibitory efficacy, which occurs during the course of the chronic diazepam treatment, is reflected by the 15% diminution of α_5-GABA$_A$ receptors in the dentate gyrus of tolerant animals. This is in keeping with the potential of a small reduction in the efficacy of GABA$_A$ receptor-mediated inhibition ($\sim$10%) to markedly increase cortical excitation (Chagnac-Amitai and Connors, 1989). Tolerance against the motor-depressant action of diazepam has been associated with an enhancement of both hippocampal synaptic efficacy and NMDA receptor subunit mRNA expression in dentate gyrus (Marin et al., 1996; Perez et al., 2003). Moreover, it has been reported that acute application of diazepam on hippocampal slices prevents the long-term potentiation of population spikes, whereas zolpidem has no effect, suggesting a possible α_5-GABA$_A$ receptor-dependent mechanism (Higashima et al., 1998). We do not exclude the possibility of a compensatory alteration in diazepam-insensitive tonic inhibition mediated by δ-subunit-containing GABA$_A$ receptors in the dentate granule cells, which could contribute to the increase in hippocampal excitability seen with chronic diazepam treatment (Nusser and Mody, 2002). However, the relatively high abundance of diazepam-sensitive α_5-GABA$_A$ receptors in the hippocampal formation positions this structure as a key player for development of the sedative tolerance phenotype by means of its widespread efferent connections to brain areas involved in the regulation of motor control.

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